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Effects of Chlordimeform on Vascular Tissue

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In order to fully understand potential risk to human beings of exposure to a chemical, one needs to understand the effects of that chemical upon each organ system and to determine its mode of action. For some classes of pesticides their primary mode of toxicity has been established. An example of this is the organophosphorus cholinesterase inhibitors. There is general agreement that their acute lethality and most of their toxicities result from cholinesterase inhibition and subsequent acetylcholine accumulation. Even among this group of compounds, however, there are toxic effects which seem to be due to other actions.

For other classes of compounds much less is known of their mechanisms of lethality and effects on organ systems. An example of this is the formamidines, the first useful one of thich was chlordimeform. Little information on the mode of action of chlordimeform was available, and on its other effects. This study was initially proposed to determine vascular actions of chlordimeform. The project was expanded to examine the cardiovascular actions of other available formamidines including formamidine metabolites which also retained the formamidine structure.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The use of pesticides over the last several years has reduced human and animal disease and increased crop yields. Although alternate pest control measures are being used to an increasing extent, it appears that the need for chemical measures for controlling pests will continue strong for the forseeable future. Many of the mainstays of chemical pest control of previous years have proved to pose to great a risk to human health and have been removed from distribution. This has of course increased useage of other pesticides and increased the urgency of finding more specific pesticides which are less toxic to humans and other non-target organisms.

One group of pesticides with a good deal of specificity (as acaricides) and seemingly low acute toxicity to higher animals is available. This type information is necessary in assuring safety of these pesticides.

F. G. Hueter, Ph.D.
Director
Health Effects Research Laboratory

PREFACE

In order to fully understand potential risk to human beings of exposure to a chemical, one needs to understand the effects of that chemical upon each organ system and to determine its mode of action. For some classes of pesticides their primary mode of toxicity has been established. An example of this is the organophosphorus cholinesterase inhibitors. There is general agreement that their acute lethality and most of their toxicities result from cholinesterase inhibition and subsequent acetylcholine accumulation. Even among this group of compounds, however, there are toxic effects which seem to be due to other actions.

For other classes of compounds much less is known of their mechanisms of lethality and effects on organ systems. An example of this is the formamidines, the first useful one of which was chlordimeform. Little information on the mode of action of chlordimeform was available, and on its other effects. This study was initally proposed to determine vascular actions of chlordimeform. The project was expanded to examine the cardiovascular actions of other available formamidines including formamidine metabolites which also retained the formamidine structure.

ABSTRACT

In this study the effects of formamidine pesticides on isolated arteries and on cardiovascular responses of the dog were determined. The mode of these actions was also investigated.

Effects of formamidine pesticides were determined in vitro on the rabbit central ear artery and aorta. Demethylchlordimeform (DCDF), formetanate and U-40481, but not chlordimeform (CDF) contracted strips of rabbit central ear artery.

DCDF caused contractions of the rabbit central ear artery in concentrations from 10^{-9} -10^{-4} M. The maximal contraction was approximately 40% of the maximal contraction to norepinephrine, and was obtained with 10^{-4} M. Neither .1 mg/ml of atropine, pyribenzamine nor methysergide affected these contractions, but .001 mg/ml of phentolamine reduced, and .1 mg/ml of phentolamine almost abolished them. These contractions were not decreased in reserpinized rabbits. Formetanate and U-40481 contracted the rabbit central ear artery with maximal contractions 22 ± 8% and 49 ± 6% of NE contractions, respectively. Maximal contractions were obtained with 10^{-3} M formetanate and 10^{-4} M U-40481 and cumulatively-added higher concentrations caused a decrease in tension from that maximum. Their contractions were antagonized by 10^{-6} M and 3×10^{-6} M phentolamine.

Three of the four formamidines antagonized contractions to vasoactive agents. CDF antagonized contractions of the rabbit aorta induced by several vasoactive agents, with the order of antagonism being potassium histamine serotonin norepinephrine. DCDF antagonized contractions of the vascular strip caused by two vasoactive agents. A concentration of 10^{-5} M antagonized contractions caused by norepinephrine and potassium but not by serotonin and histamine. U-40481 reversibly antagonized contractions induced by serotonin, norepinephrine and histamine, and to some extent potassium. Formetanate had little antagonist activity.

CDF increased the rate of ^{45}Ca washout from the medial-intimal layer of rabbit aorta, but did not affect the norepinephrine-induced decrease in ^{45}Ca washout rate. CDF also did not affect ^{45}Ca uptake by the medial-intimal strips from rabbit aorta.

Neither DCDF, U-40481 nor formetanate altered the resting rate of norepinephrine efflux from the pre-loaded rabbit central ear artery. Both U-40481 and formetanate reduced electrically-induced norepinephrine release. DCDF, applied in a lower concentration, did not alter norepinephrine release induced by either electrical stimulation or by nicotine addition.

In the anesthetized dog, CDF caused marked cardiovascular effects. These included a depressor effect on blood pressure, an initial decrease followed by an increase in musculocutaneous blood flow, and in high doses an increase in heart rate. The

mechanism of these effects does not involve stimulation of B-adrenergic nor muscarinic receptors. It enhances the vasodilation caused by isoproterenol, acetylcholine and histamine. It does not involve blockade of α or β -adrenergic, histaminergic nor muscarinic receptors, nor does it involve an inhibition of monoamineoxidase.

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LIST OF ABBREVIATIONS

ACh	-- acetylcholine
CDF	-- chlordimeform
DCDF	-- demethylchlordimeform
MAO	-- monoamine oxidase
NE	-- norepinephrine

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Sandoz Pharmaceuticals: Methysergide

The Upjohn Company: Amitraz and U-40481

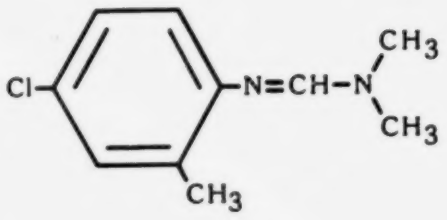
Winthrop Laboratories: l -Norepinephrine

SECTION I

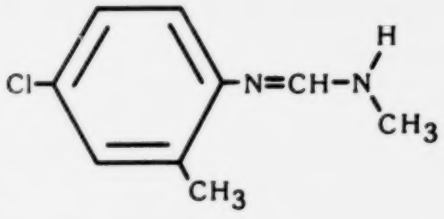
INTRODUCTION

Some chemicals containing the formamidine moiety in their structure are active as broad spectrum acaricides. The first commercially important one was chlordimeform (CDF). CDF inhibits monoamine oxidase, an enzyme involved in inactivation of norepinephrine in blood vessels as well as many other places in the body (Aziz and Knowles, 1973; Beeman and Matsumura, 1973). Although this effect does not contribute to acute lethality of chlordimeform (Robinson et al., 1975; Robinson and Smith, 1977), it was thought that it might affect cardiovascular function.

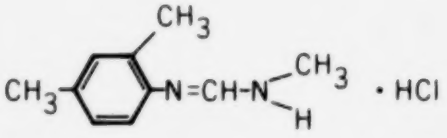
In this study the effects of several formamides on isolated rabbit blood vessels and anesthetized dogs have been investigated. The structures of the compounds investigated are below:



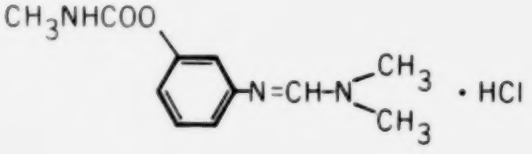
Chlordimeform



Demethylchlordimeform



U-40481A



Formetanate

CDF was the original formamidine investigated, and DCDF has been shown to be a metabolite of CDF in vivo (Knowles and Sen Gupta, 1970). U-40481 is a metabolite of amitraz (Knowles and Roulston, 1973), an acaricide which currently has Section 18 approval for use on pear trees. Formetanate is another formamidine with acaricidal activity (Knowles and Ahmad, 1971). These compounds are all available in forms which are sufficiently water soluble to allow for their in vitro study.

SECTION 2

CONCLUSIONS

This study has demonstrated that formamidine acaricides and the formamidine metabolites investigated have marked effects on isolated vascular muscle and on cardiovascular function. These actions have been quantified. The direct contractile activity of some of them in isolated arteries is due to direct stimulation of alpha-adrenergic receptors. They also interfere with contractions induced by norepinephrine, serotonin, histamine and potassium. Some of them affect calcium flux, and some interfere with electrically-induced release of norepinephrine.

In anesthetized dogs CDF affects both blood pressure and blood flow, but does not accomplish this through stimulation of the commonly known receptors. It increases the vasodilation caused by some vasoactive agents. From the in vitro studies it is thought that alteration in calcium availability may be involved in its actions.

SECTION 3

RECOMMENDATIONS

Further study should be made of the direct cardiovascular effects of formamidine pesticides in intact animals. Marked CDF-induced cardiovascular effects have been demonstrated, and other pesticides should be investigated in a similar fashion.

The marked potentiation of vasodilation in the presence of CDF should be further investigated. This probably does not involve any of the receptors usually involved in vasodilation, and may well involve calcium availability.

Effects of CDF on calcium mobilization on the cellular level should be investigated. This could be done on isolated mitochondria and sarcoplasmic reticulum preparations from vascular smooth muscle.

SECTION 4

METHODS

The effects of fomamidines on the cardiovascular system were investigated in two types of experiments. In one type, their effects on isolated arteries were investigated. These studies included evaluating their contractile activities on isolated arteries as well as their activities and selectivities at blocking contractions of other vasoactive agents. They also included a determination of their effects on calcium flux, and on norepinephrine (NE) release and reuptake.

In the other type of experiment the effects of these compounds injected into the whole anesthetized dog were investigated. In these studies the effects on blood pressure, heart rate, and musculo-cutaneous blood flow were observed.

In these studies chlordimeform (CDF) was used as a prototype. As time has permitted the effects of other foramidines have also be investigated. These include U-40481, formetanate and demethylchlordimeform (DCDF).

PROCEDURES FOR IN VITRO STUDIES IN RABBIT ARTERIES

Superfusion of Strips of Aorta Used in Chlordimeform Study

In this study strips of whole aorta (not deadventitiated) were prepared and treated as in Zelenski, et. al, 1978. Male New Zealand white rabbits were sacrificed by cervical dislocation, and the upper two-thirds of the thoracic aorta was removed and placed into physiological Ringers solution containing NaCl, 154mM; KCl, 5.4 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 6.0 mM; and dextrose, 11.0 mM in glass-distilled water. The Ringers solution was aerated with 95% O_2-5% CO_2 for at least 30 min prior to use. Excess fatty connective tissue was removed from the aorta, and it was helically cut into a strip using the method of Furchgott and Bhadrakom (1953). This strip was cut into six sections, each approximately 6 mm X 1.5 cm. Each muscle strip was tied at both ends and attached to a glass rod at the bottom and a microdisplacement myograph transducer F-50 (Narco Biosystems) at the top. Tension changes were monitored using a DMP-4A physiograph (Narco Biosystems). Tissues were suspended in a water-jacketed organ bath and superfused with Ringers solution at a constant rate using a Buchler Polystaltic pump. The superfusion and water-jacket temperatures were kept at $38 \pm 0.5^{\circ}C$.

Tissues were equilibrated at 2g tension for 60 min prior to use. After consistent contractions to submaximal concentrations of NE were obtained, the muscles were considered to be completely equilibrated. This same procedure was used on all muscles exposed to the various agonists used in this study. In preliminary experiments in which the exposure time of the strips to CDF was varied, the antagonism to the various agonists was maximal after 5 to 7 min. Seven minutes was thus chosen as the contact time of the muscle and CDF before addition of the agonist.

Responses to cumulatively added histamine were obtained on all six muscles using concentrations of 1 to 3000 μM. All concentrations were superfused for 2 min except for the 1 μ M solution which was applied for 5 min. After obtaining responses to all of the concentrations of histamine the first time, tissues were washed for 2 hr with normal Ringers solution. Four muscles were superfused for 7 min with normal Ringers solution containing 2×10^{-3} g/ml of CDF. Responses were then obtained to the same series of histamine solutions containing 2×10^{-3} g/ml of CDF. Tissues were washed with Ringers solution for 2 hr, and the procedure described above was repeated using 3×10^{-3} g/ml of CDF in the four treated muscles. Dose response curves were obtained in two control muscles each time to verify the stability of the responses over the experimental time period.

This basic procedure was also used for NE, potassium, and serotonin with minor modifications: The concentration of NE used was 10^{-10} to 10^{-4} g/ml with the muscles being exposed to each concentration for 4 min. CDF concentrations used were 2×10^{-3} and 3×10^{-3} g/ml. The concentrations of potassium were 10 to 140 mM. The osmolarity of the various potassium solutions was maintained constant by altering sodium concentration of the solutions. Muscles were exposed to each concentration of potassium for 6 min; the CDF concentrations used were 5×10^{-4} and 10^{-3} g/ml. Serotonin concentrations were 10^{-10} to 10^{-4} g/ml. Muscles were exposed to each concentration for 4 min. Concentrations used were 2×10^{-3} and 3×10^{-3} g/ml.

The effects of CDF on the contractions of aortic strips elicited by a single concentration of each of the agonists was observed using approximately equipotent submaximal concentrations of each agonist. The concentrations used were: serotonin, 10^{-6} g/ml; NE, 10^{-7} g/ml; potassium, 30 m M; and histamine, 10 μ M. Superfusion with (10^{-3} g/ml) was begun 5 min before addition of each agonist and was continued throughout exposure to the agonist. Control responses to each agonist were obtained both before and after exposure to CDF.

The CDF used in these studies was 95% pure. Most of the remaining 5% would be 4-chloro-N-formyl-0-toluidine. To determine whether the antagonism observed could be due to this impurity, studies were done using the maximal concentration of that compound which could be present in the highest concentration of CDF used with each of the four agonists. These concentrations of the impurity were without effect on contractions induced by any of the agonists.

Method of Determining Contractile Activity of Rabbit Ear Artery

Strips Used in Studies with DCDF, Formetanate, U40481A and BAAM

In these studies, central ear artery strips were used to determine agonist activity. The method is as reported in Robinson and Bittle, 1979. New Zealand White rabbits weighing 1.5 to 2.5 kg were sacrificed by cervical dislocation. The proximal half of each central ear artery was removed, cleaned, spirally-cut and divided into four sections approximately equal in length. Each strip was tied at both ends and suspended in Ringers solution (pH 7.4) in a temperature controlled muscle chamber at $38^{\circ} \pm 0.5^{\circ}$ C. The incubation solution was as previously described.

The Ringers solution was aerated with 95% 0_2-5% CO_2 for at least 30 min prior to use, and continuously while in the muscle chamber. Tension was measured using

isometric transducers and recorded by DMP-4A Physiographs (Narco Biosystems). Before all experiments, strips of artery were allowed to equilibrate under 1 g tension for 60 min with changes of bathing fluid every 15 min. Repeated additions of a sub-maximal concentration of NE were made until contractions obtained were consistent. The muscle strips were then considered to be equilibrated.

Determination of agonist activity. Contractile effects on the central ear artery were determined by the cumulative addition of 10^{-9} to 10^{-2} M formamadine to the muscle strips. Tension changes were stated as percent of tension generated to 10^{-5} M NE.

Investigation of the Mode of Agonist Action of DCDF in the Central Ear Artery

The mode of contractile activity of DCDF was investigated in the rabbit central ear artery from both normal and reserpinized rabbits as reported in Robinson and Bittle, 1979. It was observed that DCDF had considerable agonist activity in the rabbit ear artery. Experiments were designed to observe the effects of an anthihistamine (pryibenzamine hydrochloride, .1 mg/ml) an antiserotonergic (methysergide, .1 mg/ml), an anticholinergic (atropine sulfate, .1 mg/ml), and an alpha-adrenergic blocking agent (phentolamine, .001 & .1 mg/ml) on DCDF - induced contractions. In one type of experiment, responses to cumulatively increasing concentrations of DCDF were obtained, then DCDF was removed by several washings. Fifty min later one of the antagonists was added to the bath and the responses to cumulatively added DCDF were again observed.

The effects of depleting intraneuronal stores of NE on DCDF-induced contractions were also determined. This was accomplished by the intramuscular injection of 2.5 mg/kg of reserpine 72 and 24 hours prior to removing the arteries. Responses of cumulatively added DCDF were observed in muscle strips form reserpinized rabbits as previously described. Amine depletion within the strips was verified by obtaining reduced responses to nicotine.

Determination of Activity as Antagonists of Vasoactive Agents

Three kinds of experiments were designed to determine antagonist activity of the formamadines. In one type, effects on the concentration-response curve to NE, histamine, serotonin and potassium were examined. Responses to a cumulatively added vasoactive agent were obtained, and then the agonist was removed by washing. Fifty min later a formamidine was added, and 10 min after that responses to the same agonist were again determined.

In a second type of experiment, formamidine-induced effects on the response to a single concentration of the same four agonists were observed. In these studies, 10^{-6} M NE, 10^{-3} M histamine, 10^{-3} M serotonin and 40m M potassium were used as agonists. The response to one of these agonists was obtained, the drug was removed by multiple washings and 80 min later one of the formamidines (10^{-5} M) was added. After ten min contact time, the original agonist was re-added to the strip and the effects on the shape of the contraction and maximal contractile tension were observed. In a third type of experiment formamadine effects on an agonist-contracted muscle strip were examined. Concentrations of each compound were the same as in the previous study. The response to a single agonist was obtained, washed out and elicited again 90 min later. When the contractile tension was maximal, the formamidine was added and the response observed.

Reversibility of the antagonism was determined by recovery of control activity

after washing out the formamidine and re-exposing the strip to the same agonist given before the formamidine.

Determination of the Effects of Chlordimeform on Calcium Flux

Calcium efflux--

In these studies calcium flux was studied in deadventitiated aorta. Methods for CDF effects were reported in Zelenski, et al., 1978.

In this investigation, strips were prepared from the aorta as described previously, and the adventitia was removed according to the method of Maxwell et al. (1968). The deadventiated strip was then cut into two 1.5-cm strips and hung at 2 g tension in 20 ml of aerated Ringers solution. Sufficient ^{45}Ca was added to the incubation medium to make a ^{45}Ca concentration of 1.5 μCi/ml. During a 3-hr. incubation period, the Ringers solution was continuously aerated with 95% 0_2-5% CO_2 and maintained at 38 $\pm$ 0.5^{o}C.

At the end of 3 hr, the bath solution was drained, and superfusion of both muscles was started immediately with calcium-free (0-Ca) Ringers solution ($CaCl_2$,2.4 mM omitted). The superfusate was collected using a Buchler continuous automatic fraction collector Model 3T-4002 and fraction collector activator Model 3-400 8T. The rate of superfusion was 2ml/min. Samples were collected at 5-min intervals. Muscles were maintained under 2 g tension throughout the experiment.

At the end of 30 min of washing with 0-Ca Ringers solution, superfusion of one muscle with 10^{-3} g/ml of CDF was begun. The control muscle continued to receive 0-Ca Ringers solution.

After 45 min of CDF superfusion, solutions were changed for both muscles. The muscle previously exposed to 0-Ca Ringers solution containing CDF was superfused with 0-Ca Ringers solution containing CDF (10^{-3} g/ml) and NE (10μg/ml). The control muscle was superfused with 0-Ca Ringers containing 10μg/ml of NE. Samples were collected for an additional 50 min.

To determine the ^{45}Ca concentration of the superfusate samples, 0.4-ml aliquots were removed and placed in liquid scintillation minivials containing 5 ml of Aquasol-2. At the end of the washout period, muscle strips were removed, blotted, weighed, and dissolved in 0.2 ml of Soluene-100. Five milliliters of Aquasol-2 was then added to each sample. The ^{45}Ca concentration in each sample was determined using a Beckman liquid scintillation counter (Model LS-100). The counting time was automatically adjusted to obtain a counting error of less than 1%, and the counting rate of each sample was corrected for quenching.

The mean of three calcium efflux experiments was plotted as desaturation curves showing the percentage of ^{45}Ca remaining in the tissue during the continuous superfusion. (Bianchi, 1961; Weiss, 1966). Methods for studying effects on ^{45}Ca efflux of DCDF, formetanate and U-40481 were similar.

Calcium uptake - -

Methods for studying the effects of CDF on ^{45}Ca uptake were reported in Zelenski, et al., 1978. The effect of CDF on the uptake of ^{45}Ca was also determined. A deadven-

titiated aortic strip was cut into 16 approximately equal pieces weighing 5 to 10 mg each and incubated in aerated Ringers solution for 1 hr, then transferred to 0.1 mM calcium Ringers solution for an additional 90 min. Each piece was gently blotted on filter paper and placed into a minivial containing 4.2 ml of 0.1 mM calcium Ringers solution containing ^{45}Ca, 3.33×10^6 cpm. Eight of the vials contained CDF (10^{-5} g/ml). Control and exposed strips were removed at 1, 2, 5, 10, 20, 40, 60, and 90 min. Upon removal, strips were blotted on filter paper and rinsed for 5 sec in each of four successive 20-ml vol of a Tris buffer containing 10 mM lanthanum, then blotted again. (Lanthanum was used to remove extracellular calcium and to prevent the efflux of intracellular calcium.) The strips were weighed and solubilized in 0.1 ml of soluene, and their radioactivities were determined as previously described.

The significance of differences between groups was determined using Student's t test for nonpaired data, with $P < 0.05$ considered significantly different.

Methods for studying the effect of DCDF, U-40481 and formetanate on Calcium uptake were similar.

Initial calcium uptake on the addition of NE —

Ten small approximately equal sections of deadventitiated aorta were prepared from the thoracic aorta of a single rabbit as previously described in this report. A single piece was placed into a tube in Ringers solution in a shaker-heater bath for 10 minutes at 38°, then placed into another tube containing Ringers solution, ^{45}Ca, ^{14}C-mannitol, and DCDF. It was removed in 15 seconds, lightly blotted, weighed and prepared for determination of the radioactive isotopes. In a single experiment, the 9 other strips were treated in a similar manner, with a total of 5 being treated as described and 5 being exposed to the same solutions without DCDF (i.e., control). One control and one treated strip were exposed to 15, 30, 60, 120 and 300 seconds each. Mannitol was used to enable a differentiation of intracellular ^{45}Ca from true intracellular uptake. This experiment was repeated 5 times.

Similar experiments were done with U-40481 and formetanate using 5 or 6 rabbits for each drug.

Determination of the Effects on Norepinephrine Release and Uptake

Electrically-induced NE release —

The method of determining the effects of formamidines on the electrically-induced release of NE was that described for DCDF in Robinson and Bittle, 1979. A helically cut strip was prepared from the proximal 5 cm of each central ear artery as previously described. Strips from each ear were mounted between two parallel platinum electrodes for superfusion and transmural stimulation as previously described (Su and Bevan, 1970; Bevan et al., 1972), in temperature controlled organ baths (38°) at 1 g tension. The strips were incubated for 60 min in aerated Ringers solution containing .5 Ci/ml of 1-7-^3H-NE (specific activity 15 Ci/mmol). The baths were then drained and the strips superfused with Ringers solution containing 10^{-5} M NE for 2.5 min. Superfusion was continued with Ringers solution, or Ringers solution containing DCDF throughout the experiment. Stimulation periods were four min in length with 20 min between them. When the effects of 10^{-5} M DCDF were to be observed, it was added to the superfusate 8 min before electrical stimulation and maintained in the superfusate for the duration of electrical

stimulation. The superfusate was collected at 4 min intervals and an aliquot was removed for radioactivity determination by scintillation spectrometry. Radioactivity at each time period was expressed as percent of the radioactive efflux during the experiment starting with the third collection period through the end of the experiment (100min). Eight strips from 4 rabbits were used in the study.

Similar studies were done with U-40481 and formetanate using 5 and 6 rabbits.

Nicotine induced release of NE —

Effects of DCDF on nicotine-induced NE release were described in Robinson and Bittle, 1979. Effects of DCDF on nicotine-induced contractions were studied in an experiment similar to the above except that nicotine was added to the superfusate for 2.5 min every 30 min, timed so that nicotine addition and collection of a 4 min sample would begin simultaneously. Maximal tension generated by the NE released by nicotine was calculated as a percent of the contraction to 10^{-5} M NE.

Effects on Uptake of NE —

Effect of the formamidines on the uptake of ^{3}-H-NE were determined in the rabbit central ear artery. Both ear arteries were removed and cut into 4 segments each as previously described in this report. They were allowed to equilibrate for 1 hour at room temperature and transferred to a shaker-heater bath for an additional 30 min. At the time of transferring the strips to the shaker-heater bath, either CDF, DCDF, formetanate, cocaine, or U-40481 was added to the strip, or an equal volume of water (10 l) as a control. After 30 min of incubation with the various chemicals, 10^{-8} M l-^{3}H-NE was added to the bath and 60 min later the strips were removed, rinsed in 3 consecutive baths and placed into freshly aerated Ringers solution and allowed to remain there 30 min. The strips were then removed, blotted, weighed and prepared for redioactivity determination as previously described.

PROCEDURES FOR IN VIVO STUDIES IN THE ANESTHETIZED DOG

Mongrel dogs of either sex weighing between 14 and 23 kg were used. All dogs were vaccinated against canine distemper and infectious canine hepatitis, were free from internal and external parasites, and had normal hematocrit values. Dogs were anesthetized with 30 mg/kg pentobarbital i.v. and were given supplements as needed throughout the experiment. Heparin sodium was administered i.v. to prevent blood clotting. Animals that were to receive physostigmine were intubated with an endotracheal tube attached to an animal pump respirator (Harvard Apparatus).

The right cephalic vein and left femoral artery were cannulated for drug injections and for blood pressure recording, respectively, and the right femoral artery was exposed and a blood flow transducer placed around it. Musculo-cutaneous blood flow to the right hind limb, blood pressure and heart rate were constantly recorded throughout the experiment.

Experiments were designed to study the effects of CDF on the cardiovascular system of the dog, and to determine whether certain compounds would modify the responses to CDF. These compounds were the beta adrenergic receptor blocking drug propranolol, .5 mg/kg, the muscarinic receptor blocking drug atropine, 2 mg/kg, and the cholinesterase inhibitor physostigmine, 0.5 mg/kg. Appropriate test drugs were given

before and after administration of the blocking or inhibiting compounds to verify receptor blockade or enzyme inhibition. In each case the blocking agent or enzyme inhibitor was given before any CDF was administered. After blockade was established, 0.1 mg/kg of CDF was given into the cephalic vein, and washed in with normal saline. After blood pressure and blood flow rate had stabilized, a dose twice the previous dose was given and washed in with saline. This was repeated at approximately 3 minute intervals with the highest dose being 10.4 mg/kg.

In other experiments, effects of CDF on responses to other vasoactive agents were investigated. These agents were the beta-adrenergic agonist isoproterenol (Iso), 1μg/kg, the muscarinic agonist acetylcholine (ACh), .4μg/kg, the adrenergic agonist epinephrine (Epi), 2μg/kg, histamine, 10μg/kg, and the catecholamine-releasing compound tyramine (Tyr), 40 μg/kg. After responses to these substances were obtained, the series of increasingly larger doses of CDF described in the previous experiment was given. After responses to the highest dose of CDF had stabilized, responses to the test vasoactive drugs were again obtained.

Blood flow rates were determined using a Narcomatic Electromagnetic Flowmeter, Model RT-500 (Narco Biosystems, Houston, TX) and flow transducers. Blood pressure was recorded using a Pressure Transducer, Model P-1000 A (Narco Biosystems). Both blood pressure and blood flow rates were simultaneously recorded on a physiograph, DMP-4B (Narco Biosystems).

Drugs were prepared fresh each day in normal saline. Injection volume of all doses was 1 ml.

Comparisons of means for significance of differences were made using students "t" test for non paired data, except in the studies of the effects of vasoactive agents before and after CDF in the same dog. In those comparisons, the "t" test for paired data was used. $P < .05$ was considered significant.

SECTION 5

CHEMICALS

PESTICIDES

Chlordimeform, N' - (4 Chloro- 0 -tolyl) - N, N-dimethyl formamidine, was supplied as the hydrochloride by Ciba-Geigy Agricultural Chemicals.
Demethylchlordimeform, N - (4-chloro- 0 -tolyl)- N -methylformamidine, was supplied as the hydrochloride by Nor Am Agricultural Products, Inc.
Formetanate, N, N-dimethyl- N -[3-[[(methyl-amino) carbonyl] oxy] phenyl] methanimidamide, was supplied as the hydrocholoride by Nor Am Agricultural Products, Inc.
U-40481, N-methyl-N-2, 4-xylyl formamidine was supplied as the hydrochloride (U-40481A) by the Agricultural Division of the Upjohn Company.

CHEMICALS OTHER THAN PESTICIDES

The following chemicals other than pesticides used in this study and the companies which manufactured them are listed below.

Acetylcholine Chloride (Sigma)
Aquasol-2 (New England Nuclear)
Ascorbic acid (Baker)
Atropine sulfate (Sigma)
^{45}Ca, specific activity, 12 m Ci/mg (New England Nuclear)
l -Epinephrine (Eastman Organic)
Heparin sodium (U.S. Biochemical Corp.)
Histamine dihydrochloride (Sigma)
Isoproterenol hydrochloride (Sigma)
^{14}C-mannitol, specific activity 24 Ci/mM (New England Nuclear)
Nicotine (Aldrich)
l -norepinephrine-d-bitartrate (Winthrop Laboratories)
^{3}H- l-Norepinephrine hydrochloride, specific activity, 15 ci/mmol (Amersham)
Phentolamine hydrochloride (Ciba Pharmaceuticals)
Physostigmine sulfate (Sigma)
Propranolol hydrochloride (Ayerst Laboratories)
Pyribenzamine hydrochloride (Ciba Pharmaceuticals)
Reserpine, as Serpasil for injection (Ciba Pharmaceuticals)
Serotonin creatinine sulfate monohydrate (Regis)
Tyramine hydrochloride (Sigma)

Weights of all chemicals used refer to the form listed above. Water was glass distilled. Miscellaneous chemicals used were obtained from standard sources for laboratory chemicals.

SECTION 6

RESULTS

EFFECTS OF FORMAMIDINES ON RABBIT ARTERIES IN VITRO

Direct Agonist (Contractile) Activity

DCDF, Formetanate and U-40481 (but not CDF) all caused contractions of the rabbit ear artery.

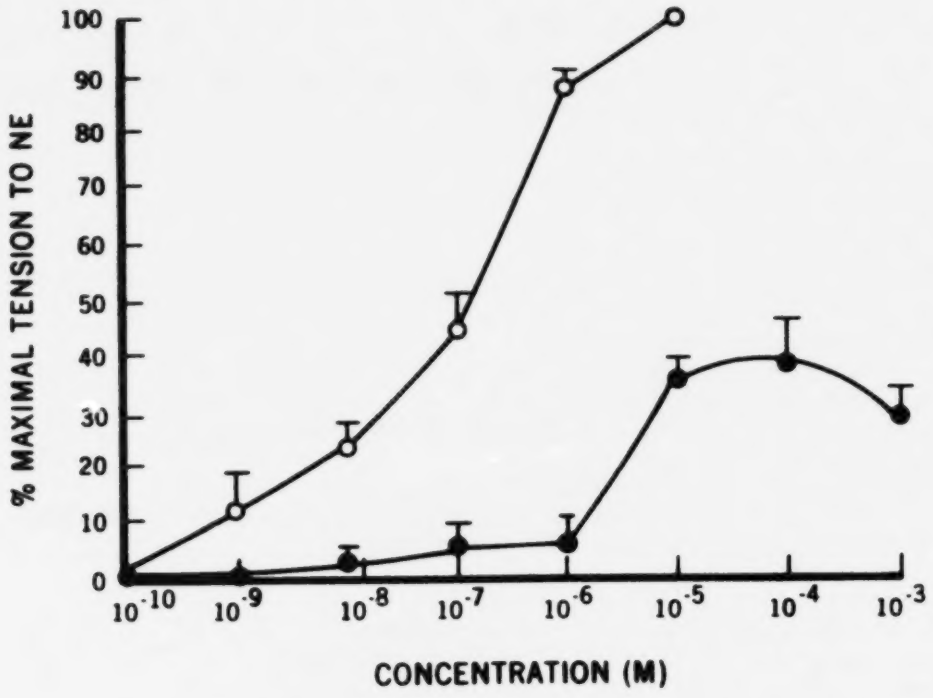


Fig. 1. Effect of norepinephrine (○) or demethylchlordimeform (●) on the contractile tension of strips of rabbit central ear artery. Each value represents the Mean ± SEM of 26 or 28 observations. (From Robinson and Bittle, 1979).

DCDF- -

Effects of DCDF as an agonist were reported in Robinson and Bittle, 1979. Cumulatively added DCDF increased contractile tension of strips of rabbit central ear artery in concentrations from 10^{-9} to 10^{-4} M (Fig 1). Maximal tension was usually observed at 10^{-4} M with the addition of higher concentrations often resulting in a decrease in contractile tension. Concentrations of 10^{-2} M or higher almost always relaxed the muscle strips to below resting tension. Maximal tension generated in response to DCDF was 39% of the maximal NE contraction. The EC_{50} for contracting the strips was 4×10^{-6} M for DCDF compared to 10^{-7} M for NE.

Formetanate and U-40481- -

Effects of Formetanate and U-40481 as an agonist are reported together. (Robinson, 1979).

Low concentrations of both formamidines increased contractile tension of ear artery strips and high concentrations decreased their tension (Fig. 2, 3). U-40481

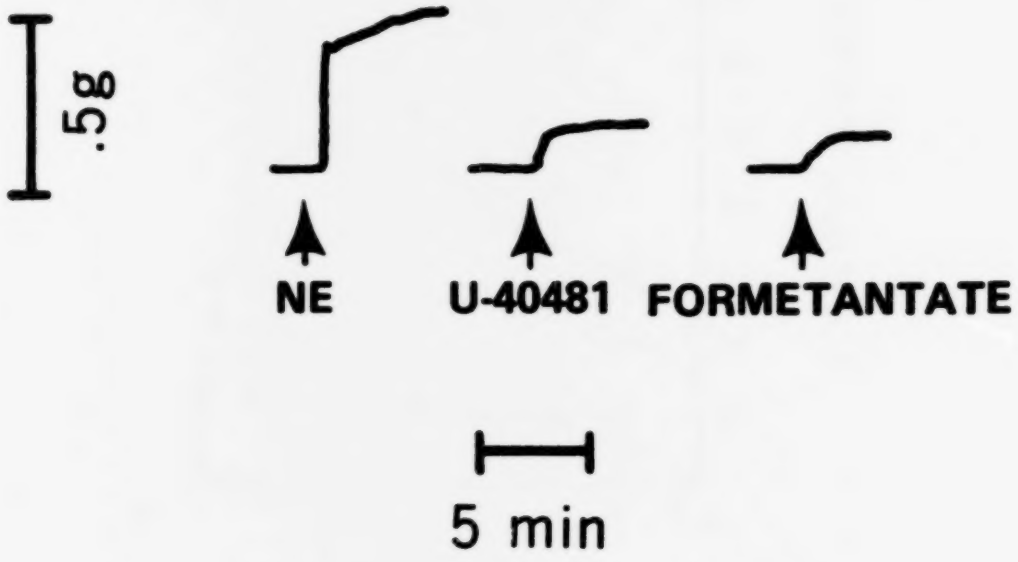


Fig. 2. Contractile response of the rabbit central ear artery in response to 10^{-5} M norepinephrine (NE), 10^{-3} M formetanate (Form.), and 10^{-4} M U40481. (From Robinson 1979).

achieved a maximal contractile tension of 49 ± 6% of the maximal tension to 10^{-5} M NE. Formetanate was less potent, achieving a maximal tension of 22 ± 8% that of NE. U-40481 elicited its maximal contraction at 10^{-4} M, while that of formetanate was obtained with 10^{-3} M. In concentrations higher than those eliciting maximal contractions, both compounds partially relaxed the strips from the maximal contraction achieved.

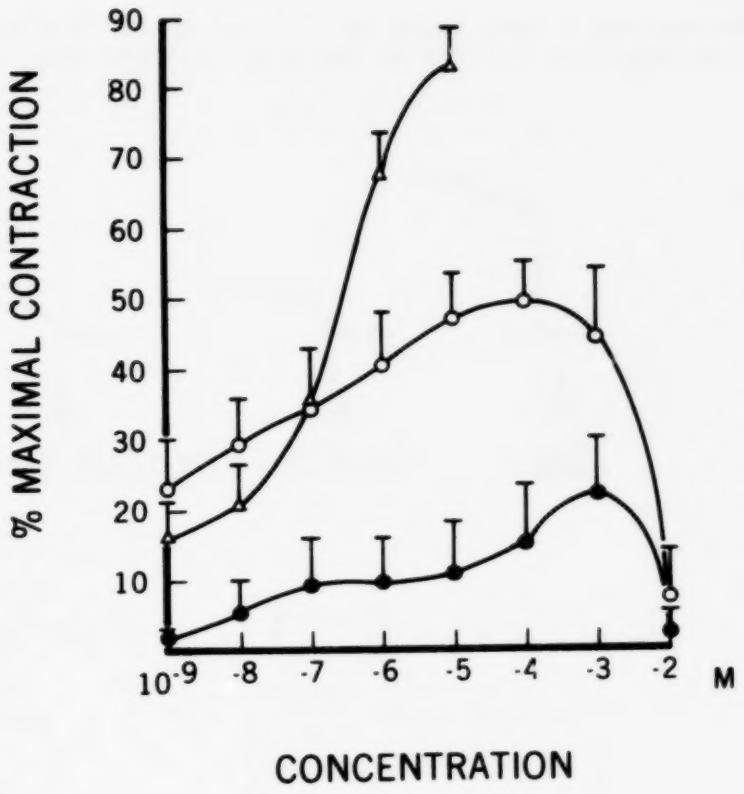


Fig. 3. Effect of NE (△) U-40481 (○) or formetanate (●) on the contractile tension of strips of rabbit central ear artery. Each value represents the Mean ± SEM of 6 observations.
(From Robinson, 1979).

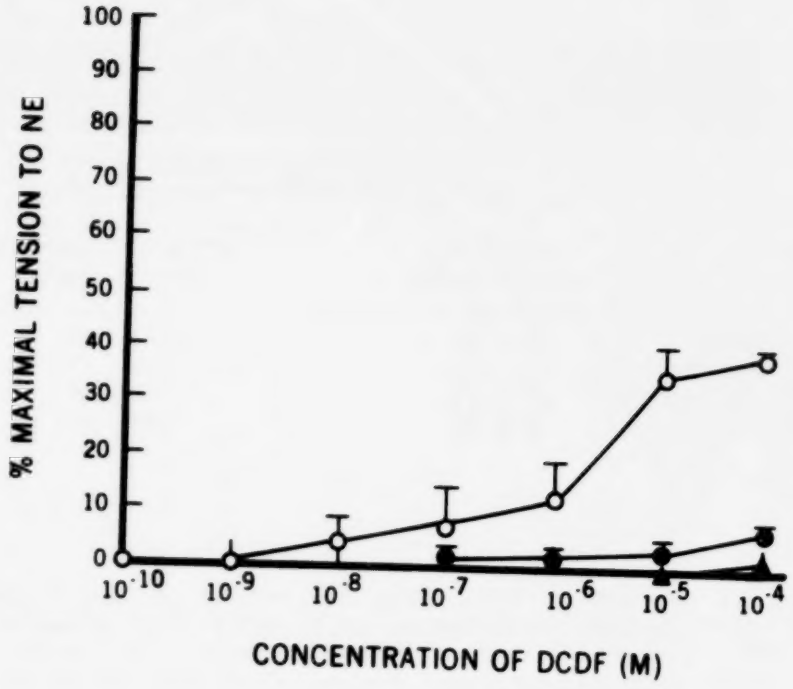


Fig. 4. Effect on the contractile tension of strips of rabbit central ear artery of demethylchlordimeform alone (○) and in strips pretreated with phentolamine. .001 mg/ml (●) or .1 mg/ml (▲). Each value is the Mean ± SEM from 5 or 7 observations. (From Robinson and Bittle, 1979).

Mechanism of the Direct Agonist Activity

Use of Selective Blocking Drugs- -

DCDF- -

Specific receptor blocking drugs were used to investigate the nature of the DCDF-induced contractions as reported in Robinson and Bittle (1979). The prior addition of either atropine, methysergide or pyribenzamine did not affect the contractions, but phentolamine greatly reduced them (Table 1). To observe the effects of phentolamine

TABLE 1

EFFECT OF PREINCUBATION OF ARTERY STRIPS WITH ATROPINE, METHYSERGIDE, PYRIBENZAMINE AND PHENTOLAMINE ON DEMETHYLCHLORDIMEFORM-INDUCED CONTRACTIONS OF STRIPS OF RABBIT CENTRAL EAR ARTERY

ANTAGONIST	CONTRACTILE TENSION (g) TO 10^{-5} M DEMETHYLCHLORDIMEFORM		
	CONTROL MEAN ± SEM	WITH ANTAGONIST MEAN ± SEM	(n)
ATROPINE, .1mg/ml	.27 ± .08	.27 ± .08	(6)
METHYSERGIDE, .1mg/ml	.21 ± .09	.22 ± .07	(8)
PYRIBENZAMINE, .1mg/ml	.31 ± .07	.28 ± .09	(5)
PHENTOLAMINE, .1mg/ml	.27 ± .03	.02 ± .01	(5)

on DCDF-induced contractions more fully, the effects of two concentrations of phentolamine on concentration response curves to DCDF were determined. In these experiments, .001 mg/ml phentolamine shifted the concentration response curve to the right, while .1 mg/ml phentolamine almost abolished DCDF-induced contractions (Fig. 2). The effect of phentolamine on the contraction induced by a single concentration of DCDF (10^{-5} M) was observed (Fig. 5a) Incubation with .001 mg/ml phentolamine reduced the contraction toadded DCDF (Fig. 5b) and with .1 mg/ml phentolamine abolished the contraction to added DCDF (Fig. 5c). In strips contracted with 10^{-5} M DCDF, the addition of .001 mg/ml phentolamine partially relaxed the strips and .1 mg/ml almost abolished induced tension within the strip (Fig. 5B). Neither atropine, methysergide, nor pyribenzamine reduced contractile tension of strips contracted with DCDF (Table 2).

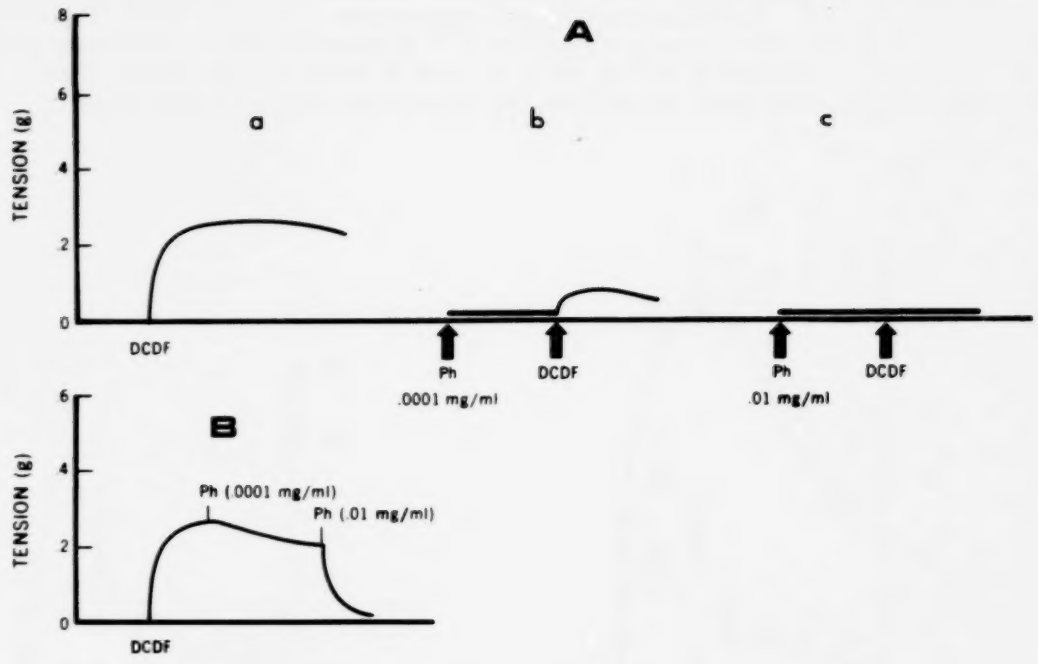


Fig. 5.A. Contraction of a strip of rabbit central ear artery caused by addition of 10^{-5} M demethylchlordimeform before (a) and after phentolamine, .001 mg/ml (b) and phentolamine, .1 mg/ml (c). B. Effect of phentolamine, .001 mg/ml and .1 mg/ml on tension of rabbit central ear artery strip contracted with 10^{-5} M demethylchlordimeform. (From Robinson and Bittle, 1979).

TABLE 2

EFFECT OF THE ADDITION OF ATROPINE, METHYSERGIDE, PYRIBENZAMINE AND PHENTOLAMINE ON STRIPS OF RABBIT CENTRAL EAR ARTERY CONTRACTED WITH DEMETHYLCHLORDIMEFORM

ANTAGONIST	10^{-5} M CONTRACTILE TENSION (g) TO DEMETHYLCHLORDIMEFORM: CONTROL MEAN ± SEM	AFTER ANTAGONIST MEAN ± SEM	(n)
ATROPINE, .1mg/ml	.40 ± .04	.40 ± .04	(7)
METHYSERGIDE, .1mg/ml	.29 ± .03	.29 ± .03	(5)
PYRIBENZAMINE, .1mg/ml	.26 ± .05	.25 ± .05	(5)
PHENTOLAMINE, .001mg/ml	.39 ± .02	.30 ± .10	(12)
PHENTOLAMINE, .1mg/ml	.39 ± .02	.00 ± .00	(12)

Formetanate and U-40481- -

Antagonism by phentolamine of contractions caused by formetanate and U-40481 are in Robinson, 1979. Phentolamine reduced contractions to both U-40481 (Fig 6A) and formetanate (Fig 6B). On the same strips 3×10^{-6} M phentolamine reduced contractions to 10^{-7} M NE to $1 \pm .8\%$ and to 10^{-5} M NE to $42 \pm 8\%$ of their control values. Thus the contractile effects seem to be caused by alpha-adrenergic-receptor stimulation.

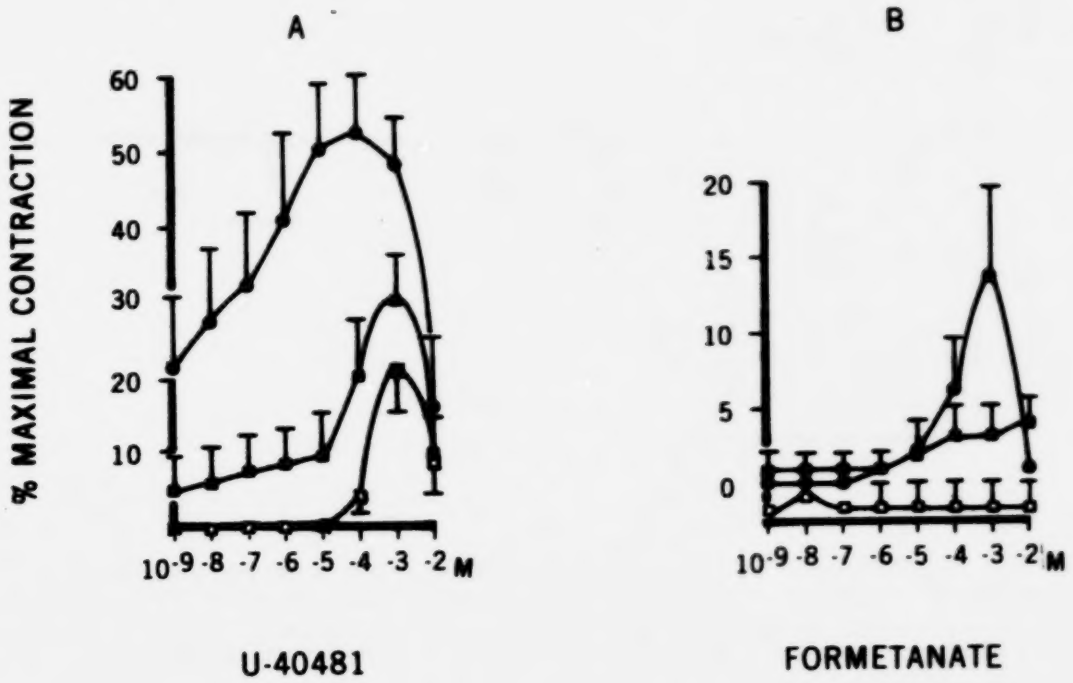


Fig. 6. Contractile tension of rabbit central ear artery strips to U-40481 (A) and formetanate (B) before (●) and during contact with phentolamine 10^{-6} M (■) and 3×10^{-6} M (□). Each value represents the Mean ± SEM of 8 observations. (From Robinson, 1979).

Use of Reserpinized Rabbits - -

Reserpinized rabbits were used to investigate the mechanism of the agonist action of DCDF as reported in Robinson and Bittle, 1979.

Rabbits that had received reserpine injections were less active than normal. Depletion of intraneuronal NE stores within the muscle strips was demonstrated by a marked reduction in responsiveness of the strips to 2 mg/ml of tyramine. The sensitivity of the central ear artery to DCDF was not reduced in arteries from reserpinized rabbits (Fig. 7). Thus contractions caused by DCDF are not dependent on NE stores.

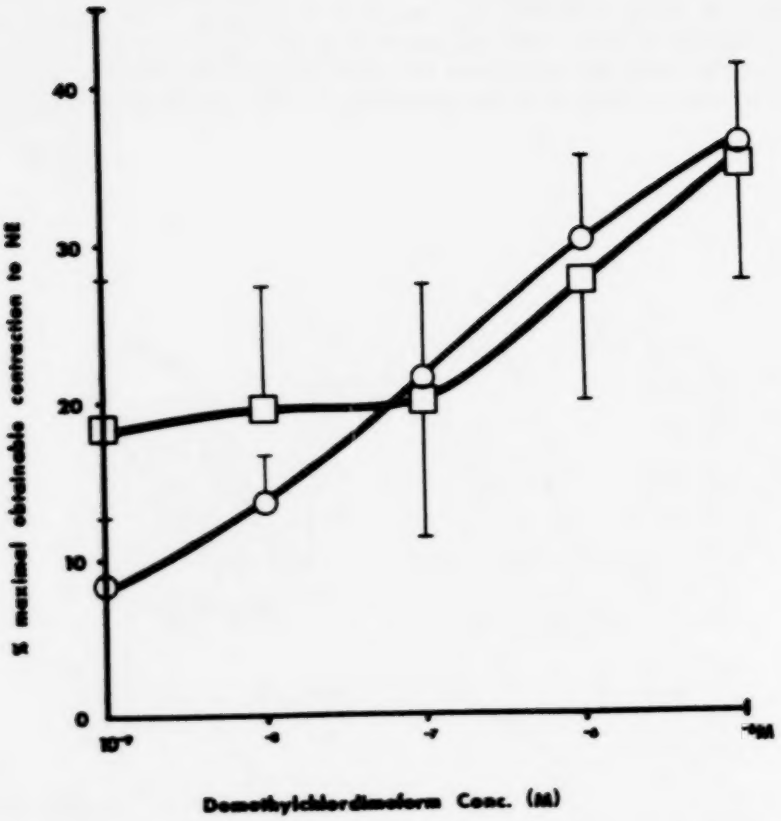


Fig. 7. Responses to demethylchlordimeform of rabbit central ear artery strips from control (○) and reserpinized rabbits (□). Each value is the Mean ± SEM from 6 or 7 observations. (From Robinson and Bittle, 1979).

Activity at Antagonizing Contractions Elicited by Vasoactive Agents

CDF- -

Effects of CDF on contractions elicited by vasoactive agents were reported in Zelenski et al. (1978). Figure 8 shows the effects of two concentrations of CDF on log concentration-response curves obtained with serotonin (Fig. 8A), NE (Fig. 8B), potassium (Fig. 8C), and histamine (Fig. 8D). CDF caused a parallel shift to the right in the NE and serotonin concentration-response curves. Although a response was elicited in control muscles at a concentration of 10^{-9} g/ml of NE of 10^{-8} g/ml of serotonin, no response was seen to NE after exposure to CDF until a concentration of 10^{-7} g/ml of NE or 10^{-6} g/ml of serotonin was applied. The maximal responses to NE or serotonin obtained in control

curves were not achieved in the presence of CDF. However, there was no indication that the tension in these muscles had reached a plateau. Potassium and histamine concentration-response curves were not shifted to the right in a parallel fashion. After treatment with CDF, maximal responses compared to control muscles were unattainable, although maximal muscle tension in the presence of CDF had been achieved.

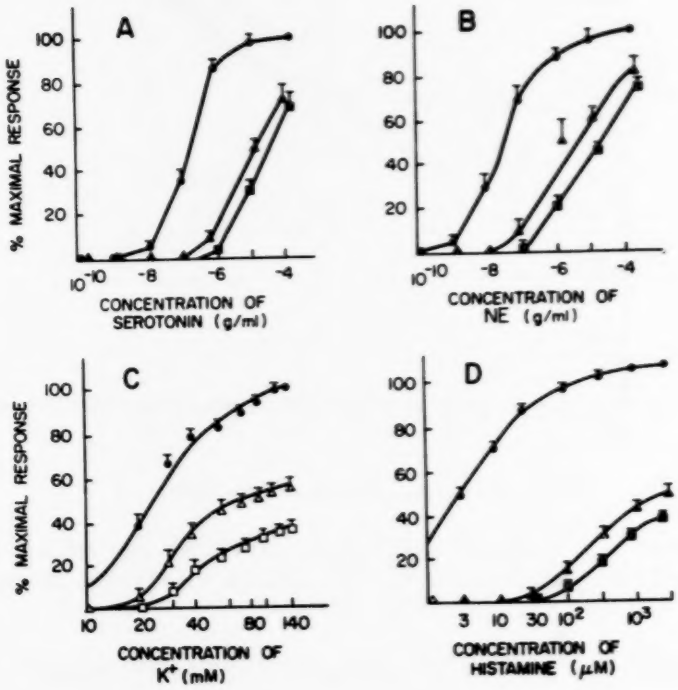


Fig. 8. Effect of CDF on contractions of rabbit aortic strips induced by serotonin (A), norepinephrine (B), potassium (C), or histamine (D). Control response (●) and in the presence of chlordimeform: 5×10^{-4} g/ml (△), 10^{-3} g/ml (□), 2×10^{-3} g/ml (▲), or 3×10^{-3} g/ml (■). (From Zelenski, et al., 1978)

The effects of CDF on contractions elicited by each of the agonists may be seen in Fig. 9. In each case, CDF caused a decrease in both the maximal tension generated and in the rate of tension development by the muscle strips. The slowed rate of tension development in the presence of CDF resulted in a change in the single tension peak in the control serotonin contraction into a double-peaked response in each of six muscles (Fig. 9A). This same effect of CDF on the shape of the response was also seen in some but not all of the responses to NE (Fig. 9B). A single-peaked response after exposure to CDF was observed in both potassium-and histamine-induced contractions (Figs. 9C and 9D).

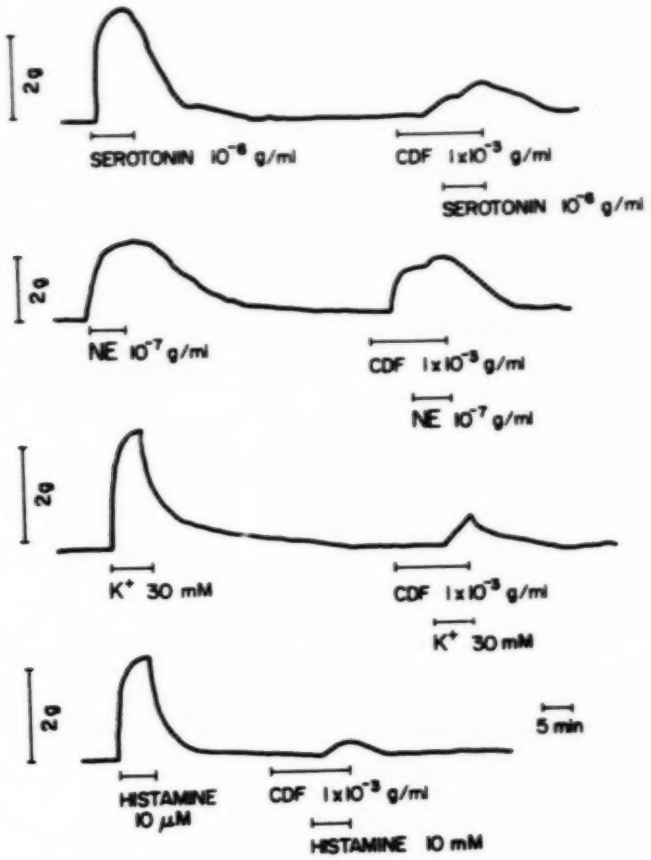


Fig. 9. Isometric recordings of the effect of CDF on responses of aortic strips to agonists. Drugs perfused during periods indicated (⊢⊣); at other times perfused with physiological solution only.

The addition of chlordimeform to muscle strips contracted by cumulatively added agonists resulted in a decrease in contractile tension in each case (Fig. 10). The initial decrease in tension was more rapid than that observed when the agonist was removed in the absence of CDF. Tension continued to decrease to almost baseline in muscles contracted by serotonin (Fig. 10A), potassium (Fig. 10C), and histamine (Fig. 10D). However, in the presence of NE (Fig. 10B), muscles relaxed an average of only 48% (range, 41-61%) within the 2-hr observation period.

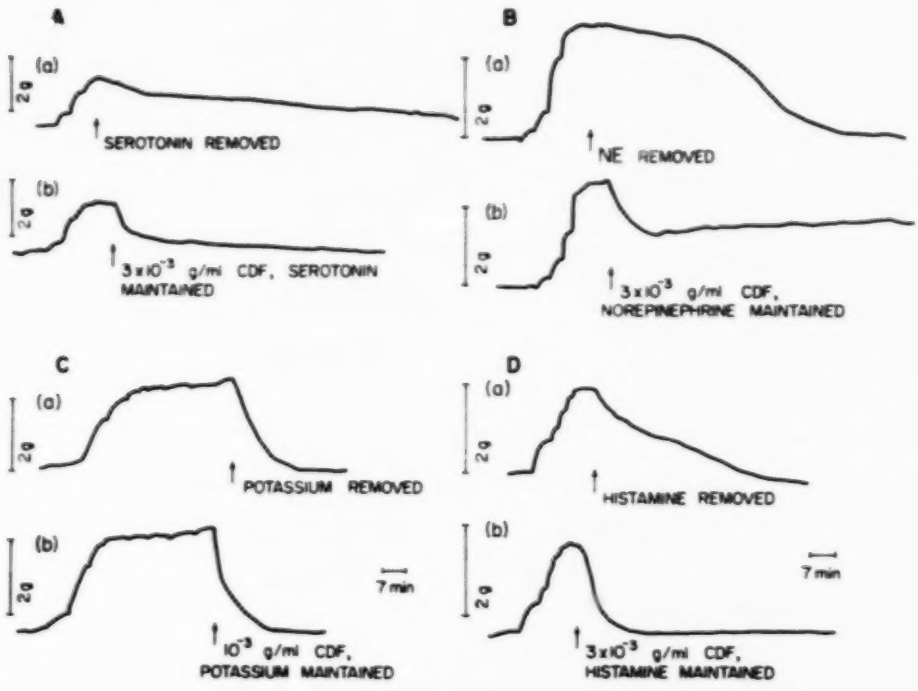


Fig. 10. Isometric recordings of the effect of CDF on rabbit aortic strips contracted by exposure to cumulatively increasing concentrations of agonist. Drugs and final concentration of agonist are as follows: (A) serotonin, 10^{-4} g/ml; (B) norepinephrine, 10^{-4} g/ml; (C) potassium, 140 mM; and (D) histamine, 3 μM. Upper tracings show relaxation on removal of agonist (arrow); lower, relaxation on addition of CDF, 3 x 10^{-3} g/ml, (arrow) without removing agonist. (From Zelenski et al, 1978).

DCDF - -

Effects of DCDF on contractions elicited by vasoactive agents were reported in Robinson and Bittle (1979).

In experiments in which muscle strips were pre-incubated with 10^{-5} M DCDF before the cumulative addition of agonist, there was a reduction in contractions induced by NE (Fig. 11 A) and to a lesser extent to those induced by potassium (Fig. 11B). Serotonin-induced contractions were not altered by this low concentration of DCDF (Fig. 11C), and contractions to low concentrations of histamine were potentiated (Fig. 11D).

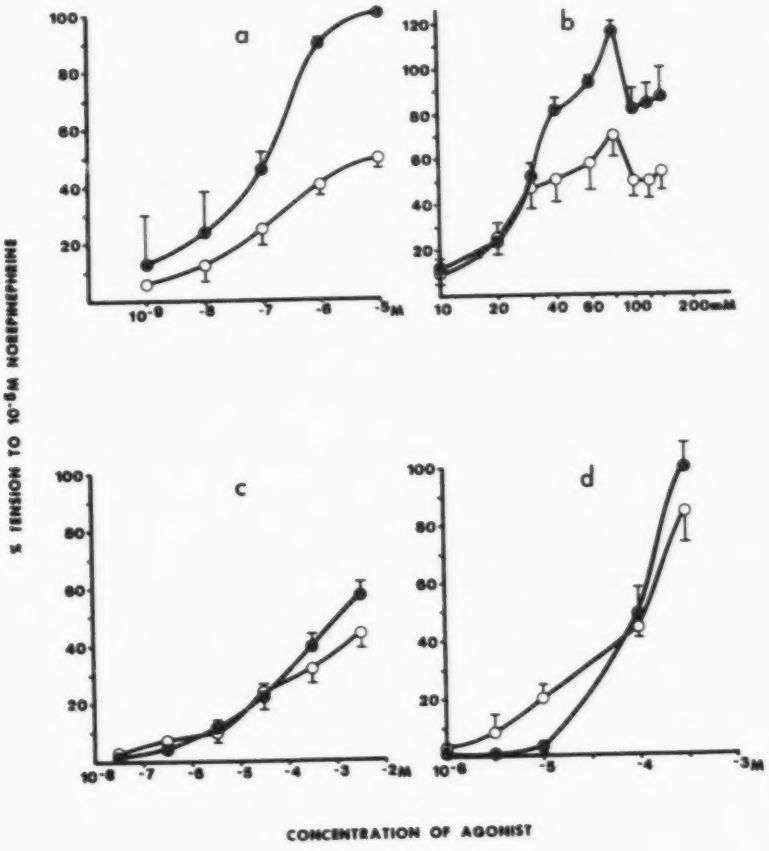


Fig. 11. Responses of strips of rabbit central ear artery to norepinephrine (a), potassium (b), serotonin (c) and histamine (d) with (○) and without (●) 10^{-5} M DCDF. Each value is the Mean ± SEM from 6-14 observations. (From Robinson and Bittle, 1979).

Formetanate and U-40481- -

Effects of formetanate and U-40481 on agonist-induced contractions are from Robinson (1979). 10^{-3} M formetanate had little effect oncontractions elicited by any of the vasoactive agents (Fig. 12). With lower concentrations of NE there was some depression of contractile responses (Fig. 12A). With lower concentrations of histamine (Fig. 12B) and through much of the curve to serotonin (Fig. 12C), contractile tension was higher than control levels. The concentration response curve to potassium was little affected by formetanate (Fig. 12D). 10^{-3} M U-40481 had much more marked effects on responses to three of the agonists.

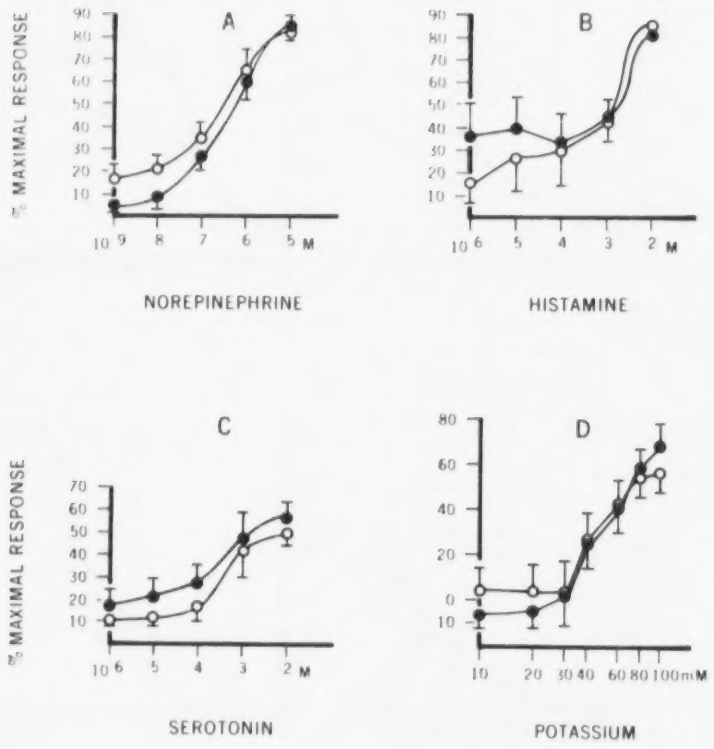


Fig. 12. Contractile tension of rabbit central ear artery strips induced by norepinephrine, histamine, serotonin, or potassium in the absence (○) or presence (●) of 10^{-3} M formetanate. Each value represents the Mean ± SEM from 5-8 observations. (From Robinson, 1979).

Responses to NE (Fig. 13A), histamine (Fig. 13B), and serotonin (Fig. 13C) were all markedly depressed by U-40481. Effects on potassium contractions were much less, with lower concentrations eliciting somewhat higher contractions in the presence of U-40481, and higher concentrations eliciting slightly depressed responses (Fig. 13D).

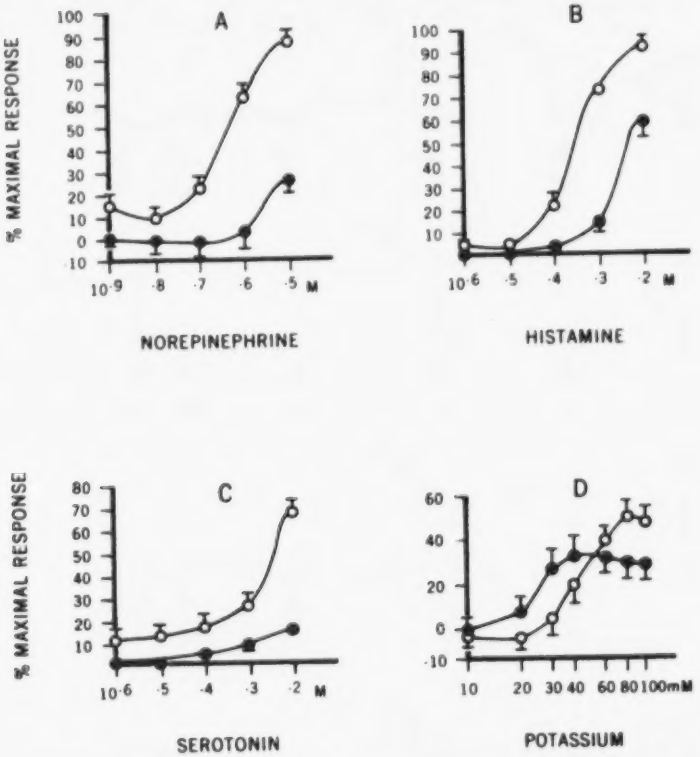


Fig. 13. Contractile tension of rabbit central ear artery strips induced by norepinephrine, histamine, serotonin, or potassium in the absence (○) or presence (●) of 10^{-3} M U-40481. Each value represents the Mean ± SEM from 6-10 observations. (From Robinson, 1979).

The addition of U-40481 to strips contracted by 10^{-6} M NE caused a rapid reduction (Fig. 14A, center) of 68 ± 20% of muscle tension. On serotonin-contracted strips there was a rapid reduction (Fig.13C, center) in tension almost to baseline (97 ± 37%). Histamine-contracted strips were also rapidly affected, but were reduced only 31 ± 6% (Fig.14B center). Potassium contractures were sometimes slightly but slowly reduced. The mean effect was a reduction of 19 ± 10% in tension.

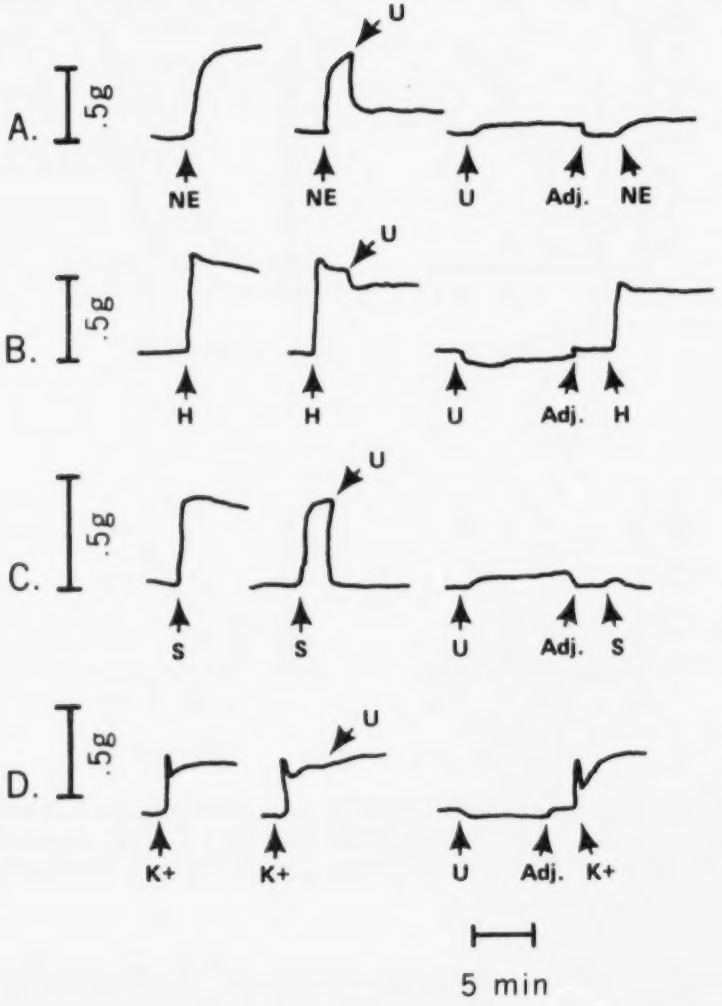


Fig. 14. Contractile tension of rabbit central ear artery strips to 10^{-6} M norepinephrine (A), 10^{-3} M histamine (B), 10^{-3} M serotonin (C), or 40 mM potassium (D) and the effect of 10^{-3} M U-40481 on the contractile tension. Left, control contraction. Center, effect of U-40481 added to contracted strip. Right, effect of presence of U-40481 on contractions to added agonist. Tension adjusted to baseline at Adj. during contact to U-40481 before exposure to agonist.(From Robinson, 1979).

Preincubation of U-40481 with muscle strips greatly diminished responses obtained to added NE (Fig. 14A, right), serotonin (Fig. 14C, right), and to a lesser extent histamine (Fig. 14B, right). Total tension induced in strips by potassium in the presence of U-40481 was not always depressed but the normally biphasic responses were separated more clearly into two components (Fig. 14D, right) by a slowing of the second phase.

Effects of Formamidines on Calcium Flux

Calcium Efflux

Effects of CDF on the efflux of 45Ca was reported in Zelenski, et al., 1978.

Figure 15 illustrates the ^{45}Ca efflux rates during superfusion with 0-Ca media containing NE, CDF, or a combination of both. The upper desaturation curve is from control muscles. The first part depicts a normal rate of calcium efflux during an initial 70-min washout with the 0-Ca medium. The latter part of the control curve shows that NE (10 μg/ml) when added to the superfusate caused a slight decline in ^{45}Ca release. The projected slope of a normal washout curve with 0-Ca Ringers solution was extrapolated.

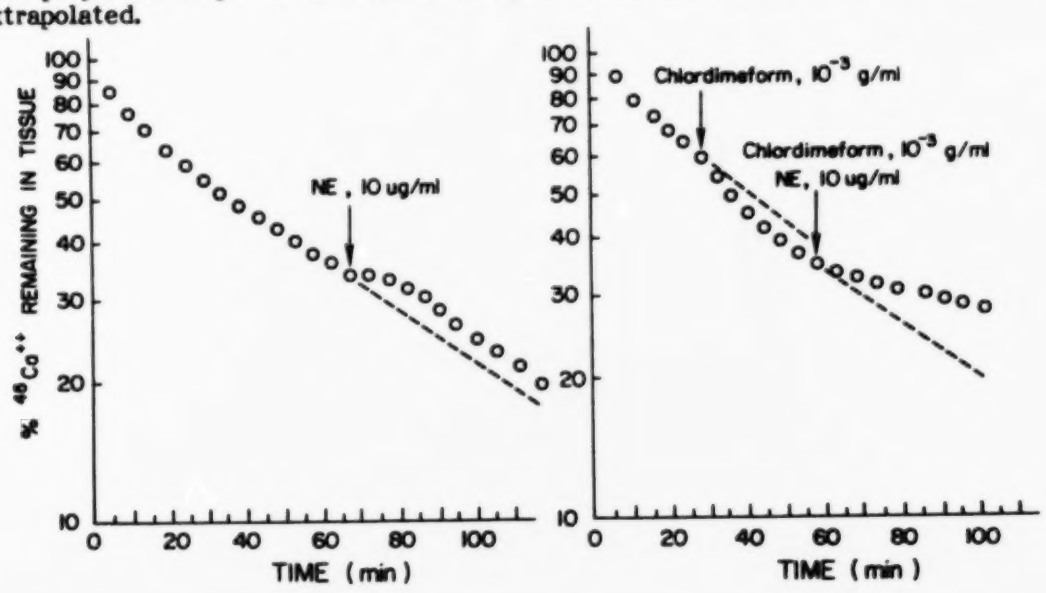


Fig. 15. Effects of CDF on ^{45}Ca efflux from media-intima rabbit aortic strips. Strips were incubated for 3 hr with ^{45}Ca prior to perfusion with calcium-free Ringers solution. Arrows indicate the addition to the perfusate of (A) norepinephrine, 10 μg/ml; (B) chlordimeform, 10^{-3} g/ml; or (C) norepinephrine, 10 μg/ml plus CDF, 10^{-3} g/ml. (From Zelenski et al., 1978)

CDF produced an initial increase in ^{45}Ca efflux which was maintained for approximately 25 min before a gradual decrease in efflux was observed. The rate of ^{45}Ca washout at this time was approximately that of the control washout curve. Addition of NE (10 μg/ml) to the Ringers-chlordimeform solution resulted in an immediate decrease in calcium efflux from the tissue. This change was even more pronounced than that observed when NE was added to the muscle strips in the absence of CDF.

Calcium Uptake

Effects of CDF on the uptake of ^{45}Ca by deadventitiated aorta strips was reported in Zelenski et al., 1978. CDF did not affect either the rate of ^{45}Ca uptake or the total uptake at 90 min in deadventitiated muscle strips (Fig. 16).

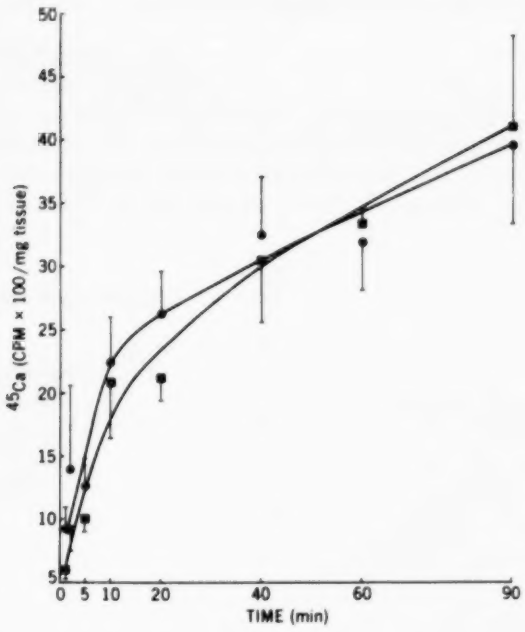


Fig. 16. Uptake of ^{45}Ca by media-intimal strips of rabbit aorta in the presence (■) or absence (●) of CDF, 10^{-3} g/ml. Each value represents the mean ± SEM of observations of strips from five or six rabbits. Values compared at each time interval were not significantly different (P 0.05). (From Zelenski et al., 1978).

Initial Calcium Uptake on the Addition of Norepinephrine

Results of the effects of DCDF, formetanate and U-40481 on the initial ^{45}Ca uptake upon addition of NE are presented in Robinson and Pento, 1979. Neither compound altered ^{45}Ca uptake significantly (Fig. 17).

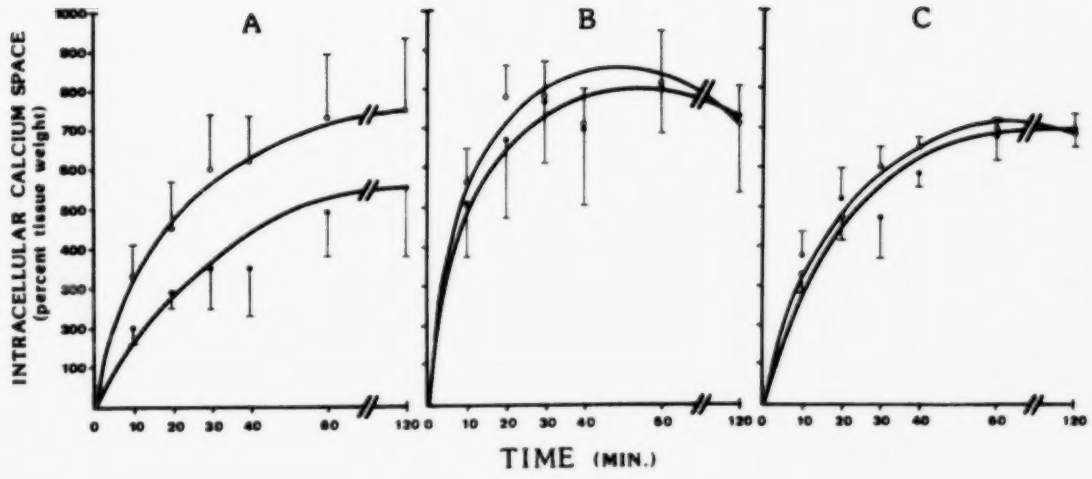


Fig. 17. Uptake of ^{45}Ca in the absence (O) and presence (●) of U-40481 (A), DCDF (B), and formetanate (C). (From Robinson and Pento, 1979).

Activity at Altering Norepinephrine Release From Sympathetic Nerves by Nerve Stimulation

DCDF- -

The effects of DCDF on electrically-induced release of radioactivity from ^{3}H-NE loaded central ear arteries is described in Robinson and Bittle, 1979. In the experiments in which helically cut strips from the central ear artery were mounted between parallel platinum electrodes and superfused with Ringers solution, the addition of DCDF did not alter ($P > .05$) the efflux of tritiated NE from the strips (Fig. 18). Transmural stimulation causes an increased release of radioactivity which decreases with each succeeding stimulation period. Prior and concurrent exposure to DCDF did not block the increased tritiated outflow from the muscle strips caused by transmural stimulation (Fig. 18).

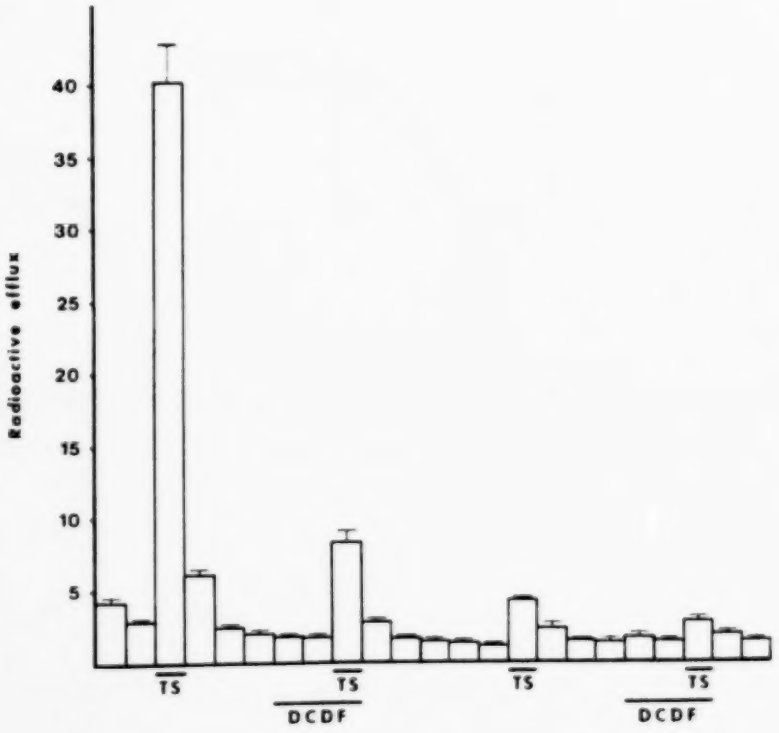


Fig. 18. Radioactivity in four-minute fractions of superfusate from ^{3}H-norepinephrine loaded rabbit central ear artery, as a percent of total radioactive efflux after initial eight minute washout. Transmural stimulation (TS) and demethylchlordimeform (DCDF) where indicated (——). From Robinson and Bittle, 1979.

Formetanate and U-40481- -

The effects of formetanate and U-40481 on electrically-induced release of radioactivity from ^{3}H-NE loaded central ear arteries is reported in Robinson, 1979. Control muscle strips pre-loaded with ^{3}H-NE, and transmurally stimulated had a progressive decrease in the radioactivity released during each of the four stimulation periods (Fig. 19A). The control strips and those treated with U-40481 were from the same rabbits, and both data were obtained simultaneously.

Neither formamidine altered the resting rate of radioactivity washout (Fig. 19B, C), but both depressed the stimulation-induced radioactive efflux. This depression of electrically-induced release was still present in the formetanate exposed strip on the second control response, despite three or more washes and a 20 min resting period (Fig. 19).

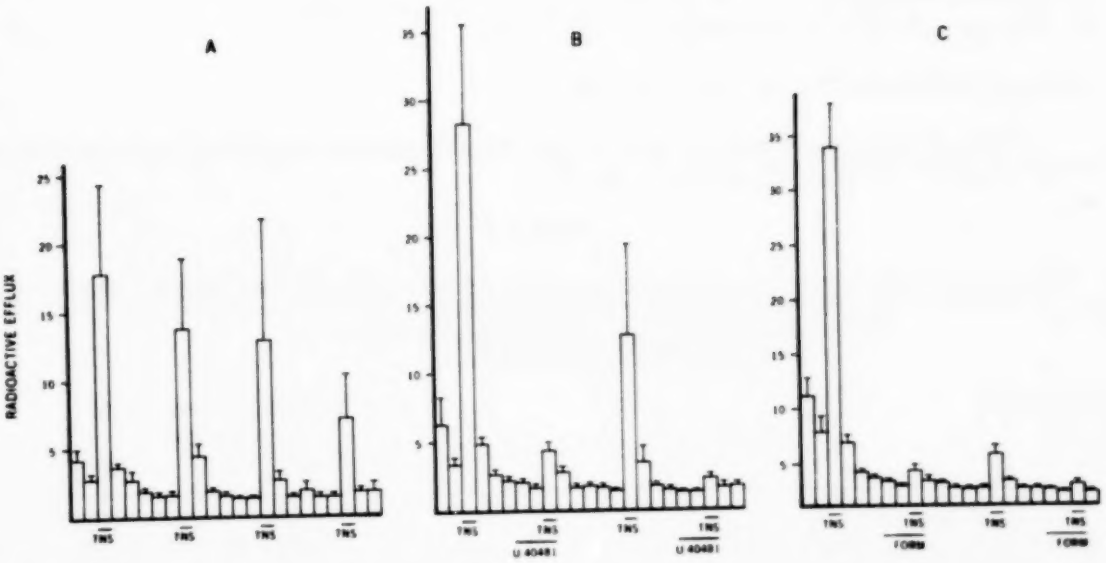


Fig. 19. Radioactivity in four-minute fractions of superfusate from ^{3}H-NE loaded rabbit central ear artery as a percent of total radioactive efflux after initial eight minute washout. Transmural stimulation (TNS) and U-40481 (in B) or formetanate (Form.) (in C) where indicated (—). Each value represents the Mean ± SEM of either 5 or 6 observations. From Robinson, 1979.

Activity at Altering Nicotine-Induced Release of Norepinephrine

Effects of DCDF on nicotine induced release of ^{3}H-NE was reported in Robinson and Bittle, 1979. In the experiments in which nicotine was added to the superfusate, the increase in tritiated efflux was not reduced (Table 3), while the nicotine-induced contractions were abolished. Thus, from the ^{3}H-NE experiments it was learned that DCDF does not alter the rate of release of NE either in the absence of releasing stimuli, or during electrical or nicotine-stimulation.

TABLE 3

EFFECTS OF DEMETHYLCHLORDIMEFORM ON NICOTINE INDUCED EFFLUX OF RADIOACTIVITY FROM AND CONTRACTIONS OF, THE RABBIT EAR ARTERY

	%Increase in Radioactive Efflux Mean ± S.E. (n)	Contraction to Nicotine[b] Mean ± S.E. (n)
Nicotine, 10^{-5} M	50.5 ± 19.1 (9)	16.1 ± 3.9 (10)
Nicotine + DCDF,	49.8 ± 34.9 (7)	.0 ± .0 (7)

a. Incubated for 60 min, in ^{3}H-NE before beginning superfusion.
b. As a percent of the contraction to 10^{-5} M NE.

Activity at Altering the Uptake of Norepinephrine

None of the formamidines tested significantly altered uptake of radioactivity by strips of rabbit central ear artery (Table 4).

TABLE 4

RADIOACTIVITY OF RABBIT EAR ARTERY STRIPS INCUBATED WITH TRITIATED NOREPINEPHRINE IN THE PRESENCE OF FORMAMIDINES OR COCAINE

Chemical	Uptake as Percent of Control Uptake Mean ± SE	(n)
Chlordimeform, 10^{-5} M	93 ± 19	(6)
Demethylchlordimeform, 10^{-5} M	83 ± 28	(5)
Formetanate, 10^{-5} M	98 ± 24	(6)
U-40481, 10^{-4} M	103 ± 31	(6)
Cocaine, 10^{-4} M	32 ± 11	(6)

9. Incubated for 30 minutes with chemicals, then 60 min with 10^{-8} M 1-^{3}H-norepinephrine.

EFFECTS OF CHLORDIMEFORM ON CARDIOVASCULAR RESPONSES OF THE DOG

Chlordimeform caused several dose related effects on the cardiovascular system. These include a fall in diastolic pressure (Fig. 20, 21A), a fall in systolic pressure (Fig. 20, 21B), an initial decrease (Fig. 20, 22A) followed by an increase in musculo-cutaneous blood flow (Fig. 20, 22B), and, in the highest dose, an increased heart rate (Fig. 23). The mean time to the maximal decrease in blood pressure following CDF injection for all doses of CDF taken together was 26 ± 2.1 sec. The mean time from the injection to the maximal initial decrease in blood pressure was 27 ± 2.0 sec. Thus the initial decrease in blood flow seems to be related to the initial fall in blood pressure.

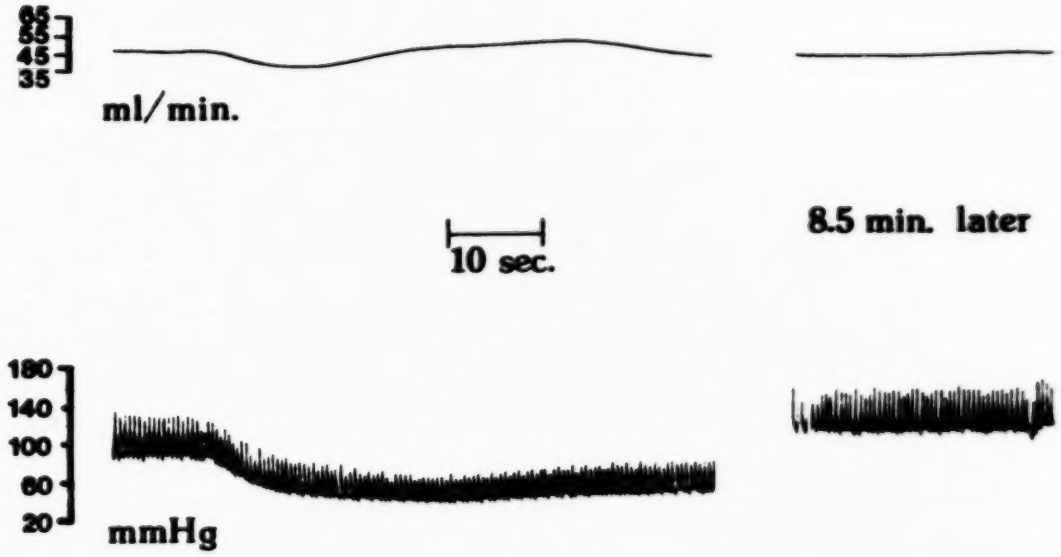


Fig. 20. Effect of 10 mg/kg chlordimeform administered 10 sec. before start of tracing on blood flow (upper tracing) and blood pressure (lower tracing) recorded from femoral arteries of the anesthetized dog. (From Robinson et al., 1979).

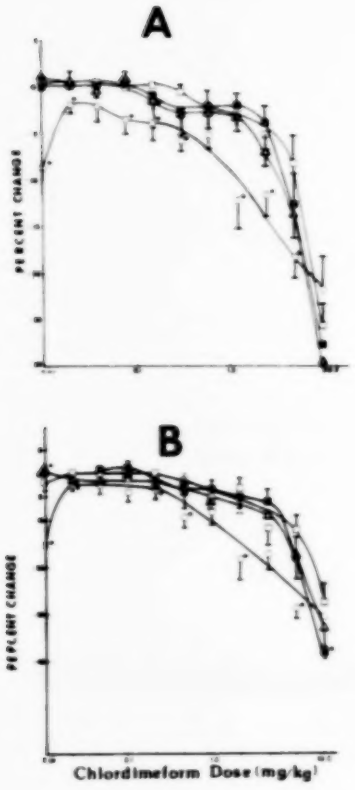


Fig. 21. Reduction in systolic (A) and diastolic (B) blood pressure following i.v. chlordimeform in dogs with no pretreatment (□), pretreatment with 2 mg/kg atropine (△),.5 mg/kg physostigmine (○) or with 15 mg/kg propranolol (■). Each value is the mean ± SEM of observations from 5 dogs. (From Robinson et al., 1979).

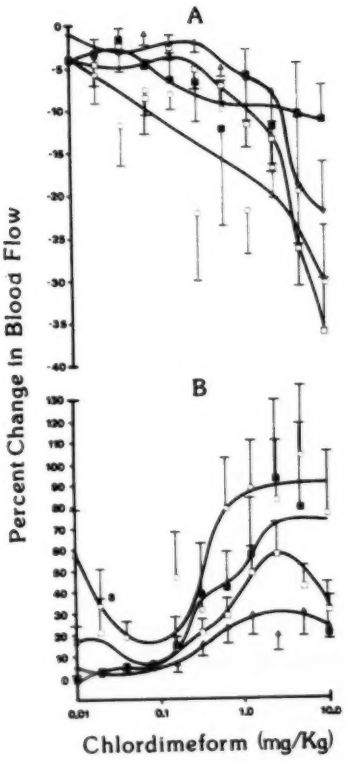


Fig. 22. Initial decrease (A) and subsequent increase (B) in blood flow in the dog femoral artery following chlordimeform. Symbols same as in Fig. 21. Each value is the mean ± SEM of observations from 5 dogs. (From Robinson et al., 1979).

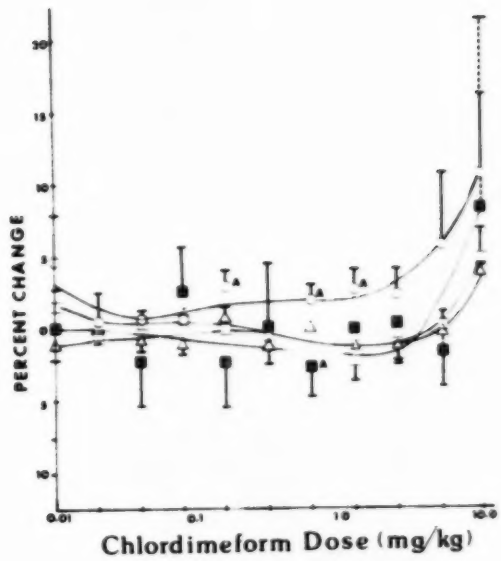


Fig. 23. Effect of chlordimeform on dog heart rate. Symbols same as in Fig. 21. Each Value is the mean ± SEM of observations from 5 dogs. (From Robinson et al., 1979).

The maximal increase in blood flow occurred 42 ± 1.9 sec. after injection of CDF and was after blood pressure had only recovered slightly (Fig.20).

Propranolol, the beta-adrenergic receptor blocking agent, was one of two receptor-blocking drugs used to investigate the mechanism of the CDF-induced cardiovascular responses. Propranolol did not significantly reduce the CDF-induced fall in blood pressure at any point, and at one point the decrease in diastolic blood pressure was even

greater in propranolol-treated dogs (Fig. 21). Propranolol reduced the initial decrease in blood flow at only the two highest CDF-doses, and did not reduce the secondary increase in blood flow (Fig. 22) nor affect CDF-induced alterations of heart rate (Fig. 23).

The muscarinic blocking drug atropine did not significantly affect CDF alterations of blood pressure (Fig. 21). At only one CDF-dose did it reduce the initial decrease in blood flow, and at only one dose did it reduce the secondary increase in blood flow (Fig. 22). It did not affect the small changes in heart rate which were caused only by higher doses of CDF (Fig.23).

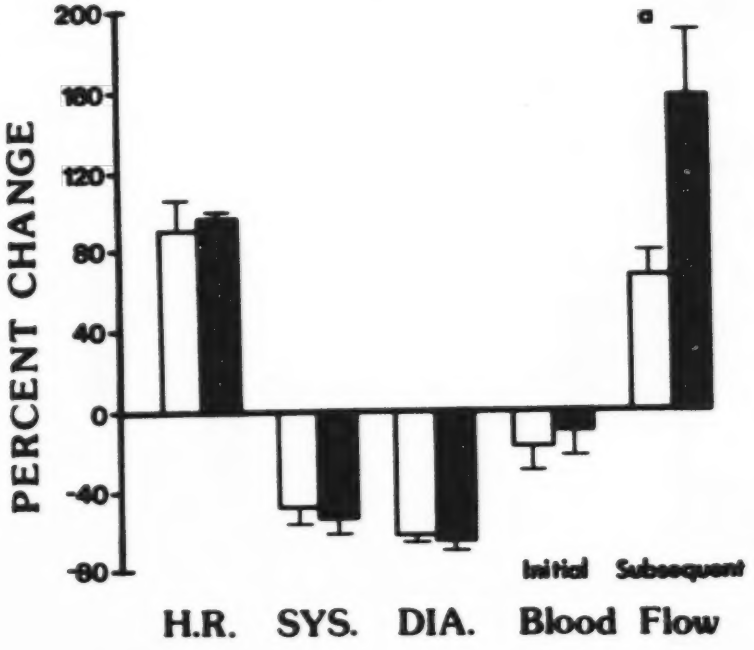


Fig. 24. Effect of 1 μg/kg isoproterenol on dog heart rate (H.R.), systolic blood pressure (Sys.), diastolic blood pressure (Dias.) and musculocutaneous blood flow before (□) and after (■) administration of 20.8 mg/kg of chlordimeform. Bar represents mean percent change ± SEM from 3 dogs. (From Robinson et al., 1979)

The cholinesterase inhibitor physostigmine-treated dogs showed greater CDF-induced effects on both blood pressure and blood flow. At several CDF doses, falls in systolic blood pressure (Fig.21A), diastolic blood pressure (Fig. 21B), and initial blood flow (Fig. 22A) and the increase in secondary blood flow (Fig. 22B) were all larger in these dogs that had received physostigmine. Physostigmine treatment caused a significantly faster heart rate following CDF treatment than was observed in control dogs.

Isoproterenol increased heart rate, decreased both systolic and diastolic blood pressure, slightly decreased initial blood flow in the femoral artery and markedly enhanced the subsequent increase in blood flow (Fig. 24). Chlordimeform altered only the increase in blood flow, increasing it from a 68% increase in control dogs, to a 157% increase in CDF-treated dogs.

The effects of ACh on blood flow were qualitatively similar to those of isoproterenol (Fig. 25). CDF effects on ACh responses also resembled those isoproterenol responses, i.e. with a significant effect only on the secondary increase in blood flow. Acetylcholine increased blood flow by 23% in control dogs and by 43% in CDF-treated dogs.

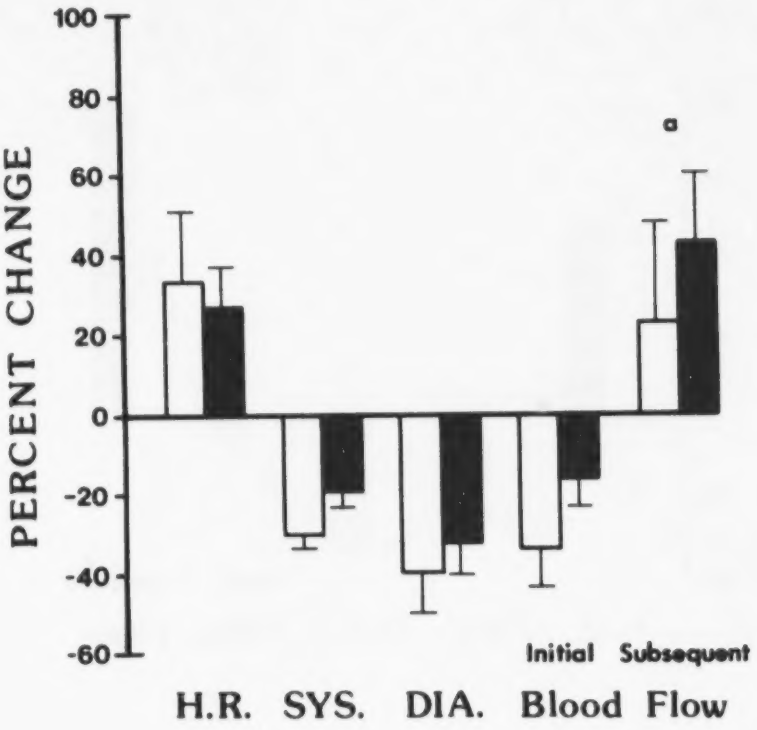


Fig. 25. Effect of .4 μg/kg acetylcholine on cardiovascular responses before and after 20.8 mg/kg chlordimeform. Symbols, etc. as in Fig. 25. (From Robinson et al., 1979).

Administered alone in a dose of 0.4 μg/kg, ACh caused a slight slowing of the heart, a fall in blood pressure, and a decrease in blood flow in the femoral artery (Fig. 26). After CDF the decreases in blood flow and systolic blood pressure were significantly different whereas heart rate and diastolic pressure were not.

Responses to epinephrine included increased blood pressure, a brief decrease in blood flow, and a secondary increase in blood flow (Fig. 26), all of which were unaffected by CDF. The compensatory slowing of the heart rate following epinephrine was blocked by CDF, with a resulting increase in heart rate.

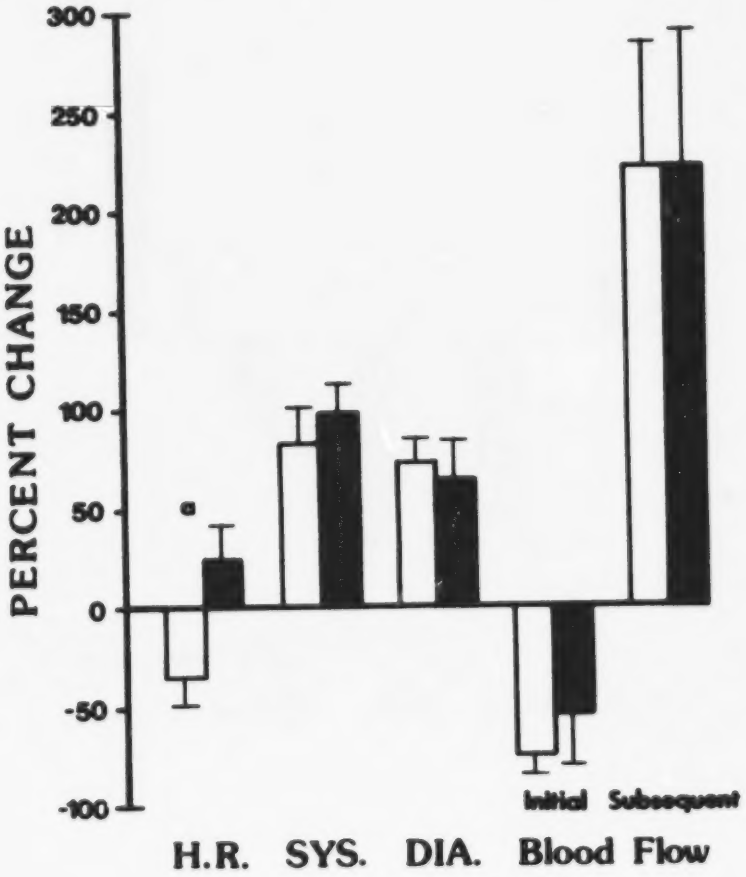


Fig. 26. Effect of 2μg/kg epinephrine on cardiovascular responses before and after 20.8 mg/kg chlordimeform. Symbols, etc., as in Fig. 25. (From Robinson et al., 1979).

Histamine also increased heart rate, decreased blood pressure, caused an initial fall in blood flow and a subsequent increase in blood flow (Fig. 27), all quantitatively similar to the effects of both isoproterenol and ACh. CDF altered only the secondary blood flow increase, causing a large increase in blood flow from 16% in control dogs to 67% in CDF-treated dogs.

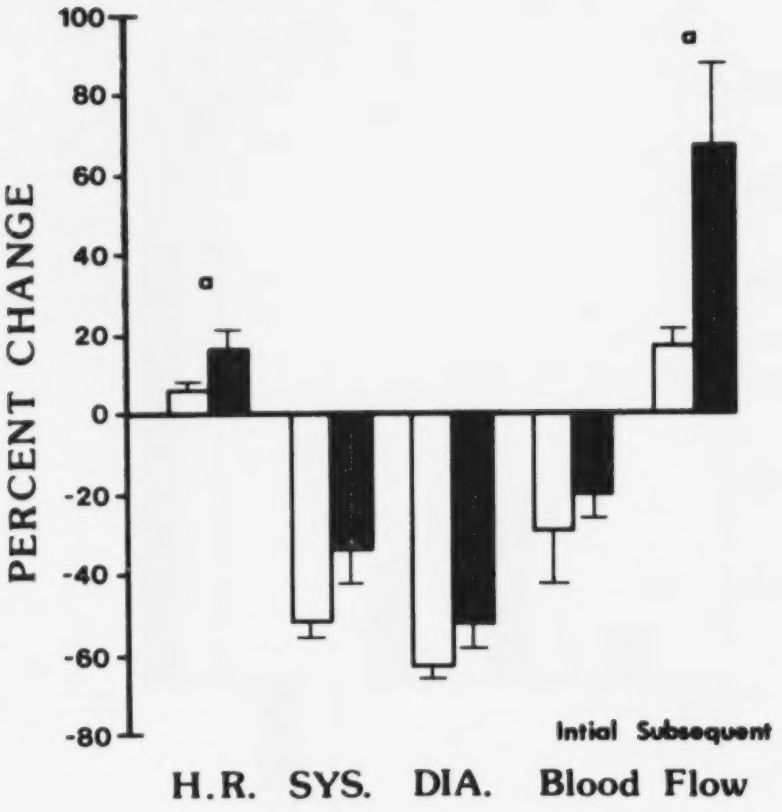


Fig. 27. Effect of 10 μg/kg histamine on cardiovascular responses before and after 20.8 mg/kg chlordimeform. Symbols, etc., as in Fig. 25. (From Robinson et al., 1979).

Responses to tyramine before CDF were as expected for a compound that releases catechlolamines, and to a lesser extent directly stimulates adrenergic receptors (Fig. 28). These effects were not altered by CDF treatment.

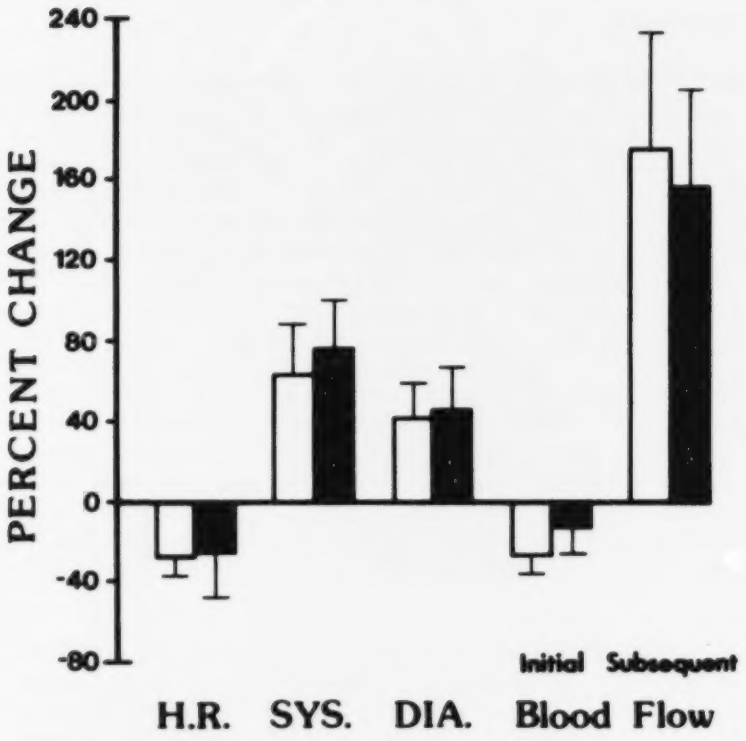


Fig. 28. Effect of 40 μg/kg tyramine on cardiovascular responses before and after 20.8 mg/kg chlordimeform. Symbols, etc., as in Fig. 2. (From Robinson et al., 1979).

SECTION 7

DISCUSSION

STUDIES IN ISOLATED BLOOD VESSELS

Actions of Chlordimeform

This is as reported in Zelenski et al., (1978), Pharmacomechanical coupling has been suggested as a name for the processes through which drugs can affect smooth muscle contraction without a necessary change in membrane potential (Somlyo and Somlyo, 1968). Contractile responses are elicited by a rise in free intracellular calcium levels, while relaxation is brought about by a fall in the cytoplasmic concentration of calcium (Bianchi, 1968). Drug-induced pharmacomechanical coupling may result, at least in part, from a rise in free intracellular calcium levels caused by an increased influx of extracellular calcium (Somlyo and Somlyo, 1968a; Durbin and Jenkinson, 1961). Activator calcium may also arise from displacement of membrane-bound calcium (Briggs, 1962). Thus druginduced contractions of smooth muscle may involve mobilization of activator calcium from two sources: one, an extracellular or perhaps loosely bound calcium; the other intracellular, membrane-bound or "sequestered" calcium (Hudgins and Weiss, 1969; Hinke, 1965). Differences in maximal responses to vasoactive agents may be due in part to either their unequal abilities to translocate calcium to contractile proteins from an intracellular store or to an unequal maximal increase in calcium permeability. In eliciting their responses, vasoactive agents seem to have considerable selectivity in the sources from which they mobilize calcium (Hudgins and Weiss, 1969, Hudgins and Weiss, 1969a; Goodman and Weiss, 1961; Adams et al., 1974; Goodman and Weiss, 1974).

It appears that CDF affects both stores of calcium but has a proportionally greater effect on extracellular or loosely bound calcium stores, if NE primarily releases bound, intracellular calcium and potassium primarily affects extracellular or loosely bound calcium as has been reported (Briggs, 1962; Hudgins and Weiss, 1968). Results of the effects of vascular strips to various agonists, on their rates of contraction after a single concentration of agonist, on their rates of relaxation after addition of CDF, and on ^{45}Ca flux are consistent with this interpretation and will be discussed individually.

CDF antagonized contractions to all four agonists examined, with the order of antagonism being potassium, histamine, serotonin, NE. The order of antagonism for potassium, histamine, and NE is that reported by Hudgins and Weiss (1969a) in decreasing order of mobilization of extracellular calcium and by Kalsner et al. (1970) in decreasing order of effect of β-diethyl-aminoethyl-diphenylpropylacetate which they judged to selectively antagonize utilization of calcium from extracellular or loosely bound sites. However, the antagonism of CDF for serotonin-induced contractions was not as great as would have been anticipated from the results of the studies of Kalsner et al. (1970) with β-diethyl-aminoethyl-diphenylpropylacetate, which they found to resemble potassium as to the pools of calcium which it affects. We are unable to explain this difference,

except that the effects of serotonin in the present study may have been somewhat potentiated by CDF inhibition of the enzyme MAO which inactivates serotonin, thus partially overcoming the antagonism of CDF. Thus the relative antagonism of CDF for NE, potassium, and histamine (but not serotonin) are in the order anticipated for an agent that interferes more with intracellular or loosely bound calcium stores.

CDF slowed contractions induced by all of the agonists. Serotonin and sometimes NE responses were thereby resolved into two peaks. The response to NE is biphasic, with the initial response probably being mediated by release of intracellular calcium and the slow phase by the release of loosely bound calcium or an influx of extracellular calcium (Brodie et al., 1959; Bohr, 1963; Sitrin and Bohr, 1971). Thus the resolution by CDF of the biphasic response to NE into two partially separated peaks must result from a slowing of the second phase of contraction.

CDF caused a rapid decrease in tension in muscles contracted by all of the agonists. Even if CDF affected only extracellular of loosely bound calcium, it would be expected to partially relax NE-induced contraction as the slow phase of contraction seems to be mediated through this calcium source (Goodman and Weiss, 1971; Kalsner et al., 1970). That NE contractures could only be partially antagonized may indicate that NE mobilized calcium from some sources unaffected by CDF.

Actions of Demethylchlordimeform

This is as reported in Robinson and Bittle, 1979. DCDF causes contractions of the rabbit central ear artery which are not reduced by blocking the muscarinic, histaminergic or serotonergic receptors, but are reduced by blocking alpha-adrenergic receptors. There are two methods by which alpha-adrenergic stimulation can occur within the rabbit central ear artery. One is by increasing the concentration at the receptor of NE from sympathetic nerve stores; the other is by a direct action on the alpha-adrenergic receptor. Among the several mechanisms whereby DCDF could increase NE concentrations at the receptor is innibition of MAO. DCDF does inhibit MAO and this could, theoretically at least, cause an increase in NE accumulation at alpha-adrenergic receptors. However, because reduction of NE stores by prior reserpinization of the rabbits did not reduce DCDF-induced contractions and because the addition of DCDF to vascular strips preloaded with tritiated NE did not affect tissue stores of NE, it thus seems to be acting directly at the alpha-adrenergic receptor as a partial agonist.

The effects of DCDF on vascular tissue have not been previously reported, but in the rabbit aorta (Zelenski, et al., 1978) as well as in the central ear artery (unpublished observations) CDF, unlike DCDF in the central ear artery, had almost no agonist activity. CDF does have agonist activity in the rectus abdominis muscle from some species of frogs (Rana nigromaculata in 10^{-3} M, and Rana Catesbiana but only in 10^{-2} M concentrations, Watanabe et al., 1975). In another species, Rana pipiens, CDF does not cause contractions of the eserinized rectus muscle in 10^{-3} M (Beeman and Matsumura, 1974). In the one species in which 10^{-3} M DCDF has agonist activity, it caused a slow contraction unaffected by d-tubocurarine (Watanabe et al., 1975). The authors speculated that the contraction may result from depolarization of the end-plate membrane, but it is apparent that it did not result from a direct stimulation of the cholinergic receptor. Thus, even in the tissue where agonist activity has been shown for the parent compound, it does not seem to result from receptor stimulation. That DCDF has agonist activity at the alpha-adrenergic receptor and the parent compound CDF does not, may seem unusual, as DCDF differs from CDF only in that a methyl group has been replaced

by a hydrogen on the terminal nitrogen. However, if this nitrogen is considered analagous to the one in epinephrine, then both DCDF and epinephrine have the same substituent groups on this nitrogen, and CDF has the same groups as does N-methylepinephrine. Epinephrine is an excellent alpha-receptor stimulant, while N-methylepinephrine, like other teniary amines of similar structure, does not stimulate alpha-adrenergic receptors.

DCDF has antagonist activity as well as agonist activity. That a partial agonist such as DCDF antagonized contractions to the full agonist NE is expected, as partial agonists are also partial antagonists. DCDF also reduced potassium contractures. The contractions of rabbit aorta to these two agonists as well as to serotonin and histamine were also antagonized by CDF in the rabbit aorta (Zelenski; et al., 1978). CDF antagonized contractions of non-vascular muscle. Watanabe et al. reported that CDF non-competitively antagonized contractions of the frog rectus abdominis muscle caused by potassium and acetylcholine (Watanabe et al., 1978), and Yamamoto and Fukami reported that it blocks at the neuromuscular junction of the larvae of the waxmoth, Galleria mellonella (Yamamoto and Fukami, 1976). In the studies on NE flux using labeled NE, there was little effect on either resting NE flux or on the amount of NE released by electrical stimulation or by nicotine. No comparable studies with either DCDF or the parent compound on NE flux have been previously reported. However, CDF did not alter the release of another transmitter, acetylcholine, in the frog sciatic nerve sartorius Muscle preparation(Wang et al., 1975).

Thus DCDF, an active metabolite of CDF, stimulates alpha-adrenergic receptors, and in high concentrations can block alpha-adrenergic and other receptors. If these actions observed at the alpha-adrenergic receptor in the central ear artery also occur at alpha-receptors within the brain, it is possible that this direct effect of DCDF may account for part of the central toxic signs (Beeman and Matsumura, 1973; Aziz and Knowles, 1973) observed after the injection of the parent compound CDF.

Actions of U-40481 and Formetanate

This is as reported in Robinson, 1979. These two formamidines, one a cholinesterase inhibiting acaricide and insecticide, the other a metabolite of an acaricide and insecticide, have several common effects on isolated arteries. One of these effects is agonist activity. Lower concentrations of both compounds cause dose-related ear artery contractures which are markedly reduced by phentolamine. Thus they both seemingly cause activation of the alpha receptor. U-40481, a secondary amine, is much more potent at this effect than the tertiary amine formetanate. DCDF also had alpha-adrenergic activity, whereas the N-methylated tertiary amine CDF did not.

Activation of alpha-adrenergic receptors does not seem to be due to release of NE because neither compound altered the resting rate of radioactive efflux from ^{3}HNE loaded strips and in fact both decreased the electrically-induced radioactive efflux.

MAO-inhibiting activity has been demonstrated for several formamidines including U-40481 (Aziz and Knowles, 1973; Beeman and Matsumura, 1973). However, it is not likely that MAO-inhibition and a resulting decreased amine metabolism contributes to the agonist activity of these compounds since acute exposure even to potent MAO inhibitors does not usually cause contractions of arterial strips.

The antagonist activities of the two formamidines also varied considerable. Formetanate had little, while an equimolar concentration of U-40481 markedly reduced

contractions ot NE, serotonin and histamine, but not to potassium. That antagonism by U-40481 to NE and serotonin contractures is similar is consistent with an earlier report that both of those amines contract the rabbit ear artery through stimulation of the same phentolamine sensitive receptor (Apperley et al., 1974).

The antagonism for histamine-induced contractions was somewhat less than for NE and serotonin contractures, and must be at a different receptor since histamine contractures do not result from alpha-adrenergic receptor stimulation (Carrol et al., 1977). Since all antagonist activities were reversible, they did not result from irreversible binding or from damaging components of the contractile mechanism.

U-40481 and formetanate-inhibition of electrically-induced release of radioactivity, may be related to local anesthetic-like activity similar to that reported for another formamidine (Chinn et al., 1976). These compounds have not been examined for that activity. On the other hand, DCDF did not inhibit electrical release but the concentration used was one-one hundreth of the one used in this study.

Structurally, U-40481 and formetanate differ in two respects. One is that U-40481 has a hydrogen and a methyl group on the terminal nitrogen, while formetanate has two methyl groups. The other is on the ring where U-40481 has two methyl groups while formetanate has a carbamyl group. It is the carbamyl moiety that confers upon formetanate cholinesterase-inhibiting activity, and it may, in addition to the extra methyl group on the nitrogen, contribute to the reduced alpha-adrenergic agonist activity compared to U-40481. This bulky group may also contribute to the relative inactivity as an antagonist that was observed with formetanate.

STUDIES IN THE ANESTHETIZED DOG

Effects of Chlordimeform

CDF markedly affected cardiovascular function in the anesthetized dog. It caused an initial decrease followed by an increase in musculocutaneous blood flow. A decrease in blood pressure was also observed at the same time the maximal increase in blood flow occurred. Thus, these were both probably caused by vasodilation. The initial decrease in blood flow may have been caused by constriction of resistance vessels of the hindlimb. This could result form central stimulation, or from peripheral actions. In the experiments on isolated rabbit arteries, although other formamidines did contract arteries directly, this was not consistantly observed with CDF. It is possible that CDF may directly constrict smaller resistance vessels initially and then cause vasodilation due to some direct effects on the blood vessels later.

Effects of Receptor Antagonists on Chlordimeform Induced Cardiovascular Changes

CDF could relax blood vessels by stimulating either β-adrenergic or muscarinic receptors found in the medial layer of blood vessels. However, because neither blockade of β-adrenergic receptors by propranolol, nor blockade of muscarinic receptors by atropine blocked the effects of CDF, it seems that CDF-induced cardiovascular effects do not involve stimulation of those two receptors. With physostigmine, however, there were some increased effects. This may indicate some involvement of muscarinic sites, or perhaps the effect is due to hemodynamic or other changes caused by physostigmine.

Effects of Cholrdimeform on Cardiovascular Actions of Vasoactive Compounds

Tyramine exerts its pressor effects through stimulation of adrenergic receptors to an extent, and to a much greater extent through release of catecholamines stored in sympathetic nerves. It is inactivated by MAO. If CDF inhibits MAO sufficiently to reduce the rate of inactivation of tyramine, then tyramine's effects should be intensified. Since tyramine's cardiovascular effects were not significantly altered by CDF, several inferences as to CDF actions can be made. One is, that CDF inhibition of MAO, even at this high dosage is not physiologically important, at least not as far as the vascular system is concerned. Another is, that CDF does not reduce the tyramine-induced release of catecholamines from sympathetic nerves. A third is, that CDF blocks neither alpha nor beta adrenergic receptors in the dog cardiovascular system. Further evidence for the lack of alpha- or beta-blocking activity was obtained with epinephrine, and for beta-blocking activity with isoproterenol.

Histamine exerts its effects by stimulating two kinds of receptors: H_1 receptors and H_2 receptors. Both are found in the cardiovascular system, with both receptors being involved in vasodilation, (Turker, 1973; Roberts et al., 1979), and probably primarily H_2 receptors involved in the stimulant action on the heart (Shimizu et al., 1970). From the data on blood flow there is no evidence of blocking the vasodilation induced by histamine, nor is there any decrease in heart rate. Heart rate in vivo following a fall in blood pressure is increased primarily by non-histaminergic mechanisms, and therefore drawing firm conclusions about histamine effects form an innervated heart in vivo is not possible.

CDF enhancement of vasodilation as demonstrated by increased blood flow following several different vasodilator drugs may be related to calcium mobilization, but no studies were done to directly study this possibility in vivo.

Thus, in summary of the study of the mode of action of the profound cardiovascular effects of CDF, we have ruled out several possible modes of action, because the effects appear not to be caused primarily by stimulation or blockade of the common vascular receptors. CDF may affect calcium availability, as CDF effects on calcium flux have been noted in the studies on isolated arteries.

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